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THE ACTIVITY OF THE PITUITARY-ADRENAL CORTEX AXIS DURING PREGNANCY AND LACTATION[1]

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THE concept of a specific pattern of systemic responses in animals subjected to nonspecific stress was originally advanced by Selye (1) in 1936. It was his opinion that the activation of the pituitary-adrenal axis was of primary importance. Many investigators have since shown that a vast number of widely varying stimuli can activate the pituitary-adrenal axis and thus enable an animal to adapt itself to the stressing agent.

Sayers *et al.* (2) formulated five classes of stressing agents which elicit increased adrenal cortical activity. They suggested that pregnancy in itself constituted one endogenous stress. Data reported by other investigators indicate that a considerable increase in adrenal cortical activity occurs during pregnancy (3, 4, 5, 6, 7, 8, 9, 10). The placenta in all probability contributes directly to this increased cortical activity by elaborating and releasing ACTH (11, 12).

Parturition is another endogenous stimulus which may normally constitute a stress condition (13, 6, 10). Bromberg, Feldman, and Eliakim (14) report that increased adrenal activity is associated with normal lactation, and suggest that the neurogenic stimulus of nursing elicits an increased release of the adrenocorticotrophic hormone. In contrast Robertson and Mixner (15) report a lower level of circulating 17-hydroxycorticosteroids in lactating than in nonlactating cows.

This investigation was undertaken to determine the relative activity of

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the anterior pituitary-adrenal cortex axis in the diestrous, pregnant, and lactating laboratory rat. Changes in adrenal cholesterol concentration were considered an index of cortical activity. Rosenfeld (16) recently demonstrated a direct relationship between the depletion of adrenal cholesterol and corticosteroid output. Sayers and Cheng (17), who first investigated the influence of stress on pituitary ACTH content, report a 35% decrease in response to scalding. More recently Sydnor and Sayers (18) failed to find a decrease of pituitary ACTH in response to stress, but did find an increase in pituitary ACTH a result of adrenalectomy. There are no published data on the effects of pregnancy and lactation on pituitary ACTH content.

MATERIALS AND METHODS

Virgin female rats derived from crossing Long-Evans and Wistar strains were used when their age was 90 days and their body weights in the range 230 ±20 gm. At least twelve rats were employed in each of the following groups: diestrus, 5th, 10th, 15th, 20th and 21st day of pregnancy, 1 and 24 hours after parturition, 5th, 10th, 15th and 20th day of lactation. Rats assigned to the group to be sacrificed one hour after parturition were placed on a raised ¾-inch mesh wire 36–48 hours before parturition to be sure they would not be suckled. Rats assigned to the lactating groups were allowed to retain their entire litters for the first 48 hours. At this time litters were standardized at 8 ±1 pups. All pups were removed from lactating rats one hour before they were sacrificed.

Rats were sacrificed at the appropriate time by placing them in sealed jars which contained volatilized ether. The pituitary glands were removed and the anterior lobes were freed and quickly weighed to the nearest 0.1 mg. on an analytical balance equipped with a reading scope. The weighed pituitary glands were placed in marked vials and frozen for subsequent ACTH determinations. The adrenal glands were then removed and carefully dissected, weighed, and placed in an extraction tube. Total and free adrenal cholesterol concentration was determined according to the method of Schoenheimer and Sperry (19) as modified by Sperry and Webb (20). The concentration of adrenal ester cholesterol was determined by difference.

Pituitary ACTH values were determined by the method of Burns *et al.* (21) as modified by Sydnor and Sayers (18). The frozen pituitary glands from each group were ground to a homogeneous powder in 0.1N hydrochloric acid. Suspensions were so made up in 0.01N hydrochloric acid that 0.6 ml. contained 200 μg. of fresh pituitary material. Assay animals were hypophysectomized rats, weighing 120–140 gm.[3] Approximately 24 ±3 hours after hypophysectomy the animals were anesthesized with sodium pentobarbital (4 mg./100 gm. body weight) and the left adrenal was removed. The pituitary suspension was administered into the femoral vein. Each assay animal received 200 μg. of fresh pituitary tissue and twelve assay animals were used to determine the pituitary potency of each experimental group. One hour after the administration of the pituitary material the assay animals were sacrificed and the right adrenal gland removed. Pituitary potency was determined by the ascorbic acid depletion technique described by Sayers, Sayers and Woodbury (22). Ascorbic acid analyses were carried out by the method of Roe and Kuether (23) as modified by Schaffert and Kingsley (24).

Mean and the standard error of the mean were calculated for all treatment groups. The significance of differences between groups was tested by Fischer's "t" test.

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[3] Obtained from the Charles River Breeding Laboratory (Brookline, Massachusetts).

TABLE 1. ADRENAL WEIGHT AND ADRENAL CHOLESTEROL CONCENTRATION DURING PREGNANCY AND LACTATION

Group	Adrenal wt., mg.	Cholesterol concentration (mg./100 mg. adrenal tissue)		
		Total	Ester	Free
Diestrus	64.4 ±2.54*	2.792 ±0.170	2.540 ±0.174	0.281 ±0.015
Fifth Day of Pregnancy	59.8 ±2.50	3.188 ±0.210	2.515 ±0.302	0.401 ±0.047
Tenth Day of Pregnancy	60.5 ±2.96	2.635 ±0.191	2.169 ±0.240	0.386 ±0.070
Fifteenth Day of Pregnancy	68.1 ±1.45	1.811 ±0.132	1.223 ±0.098	0.588 ±0.045
Twentieth Day of Pregnancy	65.9 ±2.29	2.267 ±0.128	1.915 ±0.127	0.352 ±0.041
Twenty-First Day of Pregnancy	60.6 ±2.50	2.792 ±0.185	2.470 ±0.160	0.321 ±0.030
One Hour After Parturition	62.4 ±3.16	1.375 ±0.095	1.139 ±0.103	0.237 ±0.023
First Day of Lactation	66.7 ±2.49	1.500 ±0.104	1.299 ±0.081	0.201 ±0.029
Fifth Day of Lactation	68.7 ±1.57	1.183 ±0.079	0.928 ±0.079	0.255 ±0.018
Tenth Day of Lactation	76.3 ±2.12	0.809 ±0.093	0.515 ±0.068	0.293 ±0.036
Fifteenth Day of Lactation	83.4 ±3.52	0.814 ±0.074	0.625 ±0.064	0.188 ±0.013
Twentieth Day of Lactation	77.8 ±2.81	0.972 ±0.150	0.788 ±0.135	0.184 ±0.021

* Results expressed as means and their standard errors.

RESULTS

Adrenal Cholesterol Concentration

In the diestrous rat total adrenal cholesterol concentration was found to be 2.8 mg. %. Ninety per cent of this cholesterol was in ester form and the remainder was free cholesterol (Table 1). On the 5th day of pregnancy total cholesterol was slightly higher than the diestrous values, however, an increase which was entirely in the free form. The concentration of ester cholesterol remained unchanged. A significant decrease in total cholesterol concentration was noted from the 5th to the 10th day of pregnancy, a decrease which was for the most part in the ester form. These changes are shown in Table 2.

The concentrations of adrenal cholesterol observed on the 15th day of pregnancy, which is the beginning of the last trimester of pregnancy in the

TABLE 2. CHANGES IN ADRENAL TOTAL AND ESTER CHOLESTEROL CONCENTRATION DURING PREGNANCY AND LACTATION

Between groups	Change in total μg./100 mg. adrenal wt.	Change in ester μg./100 mg. adrenal wt.
Dietrus to fifth day of pregnancy	+ 395	+ 25
Fifth to tenth day of pregnancy	− 552*	− 347
Tenth to fifteenth day of pregnancy	− 824**	− 945**
Fifteenth to twentieth day of pregnancy	+ 456*	+ 691**
Twentieth to twenty-first day of pregnancy	+ 525*	+ 556*
Twenty-first day of pregnancy to one hour after parturition	−1,417**	−1,332**
One hour after parturition to first day of lactation	+ 126	+ 160
First to fifth day of lactation	− 318*	− 372**
Fifth to tenth day of lactation	− 374**	− 412**
Tenth to fifteenth day of lactation	+ 005	+ 110
Fifteenth to twentieth day of lactation	+ 159	+ 163

* Change is statistically significant (P 0.05).
** Change is statistically highly significant (P 0.01).

rat, were lower than at any other time during pregnancy. Highly significant decreases in both total and ester cholesterol concentration were observed from the 10th to the 15th day of pregnancy. Ester cholesterol concentration increased significantly during this period. The ester cholesterol concentration on the 15th day of pregnancy was 1.2 mg.% or less than half the concentration observed in the diestrous rat. From the 15th day to the time of parturition, cholesterol concentration was found to increase.

A significant increase in total and ester cholesterol was observed from the 15th to the 20th day of pregnancy and again from the 20th to the 21st day. The concentration of total cholesterol on the 21st day of pregnancy was 2.8 mg. % or the same concentration as observed in the diestrous rat. Ester cholesterol concentration at this time was slightly lower than in the diestrous state.

Parturition was accompanied by a highly significant decrease in adrenal cholesterol. Total concentration dropped from 2.8 mg. % on the 21st day of pregnancy to 1.4 mg. % 1 hour after parturition. The decrease in ester cholesterol was even more marked. A slight increase in adrenal cholesterol concentration was observed between the groups sacrificed 1 and 24 hours after parturition although this increase was not significant. Adrenal cholesterol decreased from the 1st day of lactation to the 5th; the decrease was significant for total cholesterol and highly significant for the ester form. Highly significant decreases in total and ester forms were noted between the 5th and 10th days of lactation. The total cholesterol concentration on the tenth day of lactation was 0.8 mg. % or less than 30% of the level observed in the diestrous rat. Ester cholesterol concentration was found to be less than 20% of the diestrous level.

TABLE 3. ADRENAL ASCORBIC ACID DEPLETING CAPACITY AND ADRENAL CORTICOTROPHIN CONTENT OF THE RAT ANTERIOR PITUITARY GLAND DURING PREGNANCY AND LACTATION

Material administered	Adrenal ascorbic acid		MU of ACTH per 200 μg. of AP tissue
	Initial (mg./100 mg. adrenal tissue)	Depletion	
One milliunit (MU) of ACTH*	563.03 ± 3.45	165.50 ± 18.62	
200 μg. of anterior pituitary tissue from rats in diestrus	635.97 ± 22.03	149.32 ± 17.03	0.9022
200 μg. of anterior pituitary tissue from rats on fifteenth day of pregnancy	629.42 ± 14.24	127.88 ± 22.13	0.7727
200 μg. of anterior pituitary tissue from rats on twenty-first day of pregnancy	603.42 ± 29.01	198.81 ± 23.66	1.2013
200 μg. of anterior pituitary tissue from rats one hour after parturition	596.86 ± 24.98	121.84 ± 23.62	0.7361
200 μg. of anterior pituitary tissue from rats on tenth day of lactation	674.02 ± 55.50	157.31 ± 11.64	0.9505

* One milliunit equals one one-thousandth of a unit of U.S.P. corticotrophin reference standard.

Slight increases in total and ester cholesterol were noted from the tenth to the fifteenth day of lactation and again from the fifteenth to the twentieth. These increases were not significant.

Pituitary ACTH Determinations

The anterior pituitary glands from rats which were sacrificed in diestrus, on the fifteenth day of pregnancy, on the twenty-first day of pregnancy, one hour after parturition, and on the tenth day of lactation were assayed for their ACTH content. These groups were selected because the previous adrenal cholesterol determinations indicated that they were the periods of maximum change in adrenal cortical activity during pregnancy and lactation. Pituitary ACTH potency was determined by injecting 200 μg. of pituitary tissue into each assay animal and measuring the resulting adrenal ascorbic acid depletion. One group of assay animals was treated with one one-thousandth of a unit of USP corticotrophin reference standard for comparison purposes. The results of these assays are presented in Table 3.

The ascorbic-acid-depleting capacity of 200 μg. of pituitary tissue from rats sacrificed in diestrus was 149 mg. per 100 gm. of adrenal tissue. Ascor-

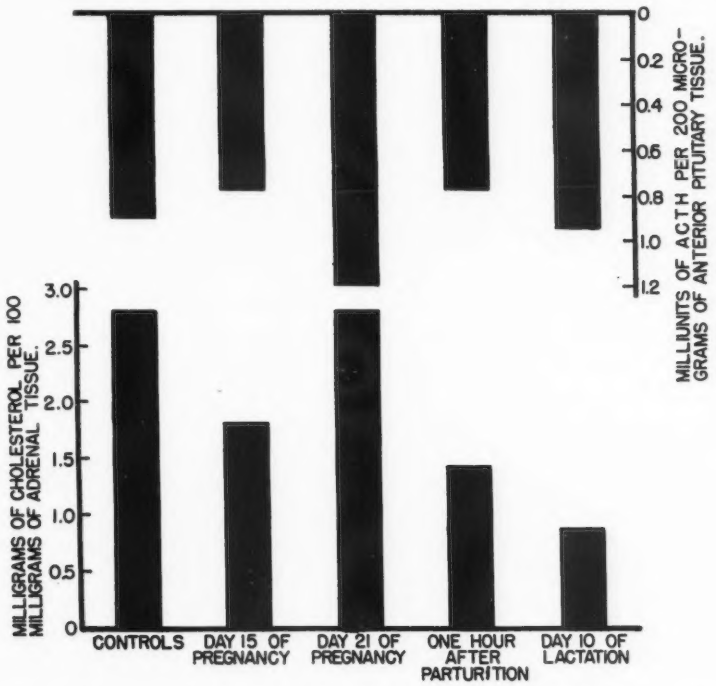

FIG. 1. Concentration of pituitary adrenocorticotrophin in relation to the concentration of adrenal cholesterol during pregnancy and lactation.

bic acid depletion caused by the pituitaries of rats sacrificed on the fifteenth day of pregnancy was 13% lower than in diestrus or 128 mg. per 100 gm. of adrenal tissue. An increase in ascorbic-acid-depleting capacity was noted between animals sacrificed on the 15th and 21st day of pregnancy. The value for the pituitaries of rats sacrificed on the 21st day of pregnancy was 199 mg. per 100 gm. of adrenal weight. A decrease in adrenal ascorbic-acid-depleting capacity was observed between the pituitaries of animals sacrificed on the 21st day of pregnancy and those sacrificed 1 hour after parturition. The pituitary potency of rats sacrificed 1 hour after parturition was 122 mg. per 100 gm. of adrenal tissue; however, by the tenth day of lactation this potency had increased to 157 mg. per 100 gm. of adrenal tissue.

When the data for pituitary ACTH concentration are compared with the data for adrenal cholesterol concentration it is noted that changes in ACTH concentration are associated with changes in adrenal cholesterol concentration. Moreover, with the exception of the tenth day of lactation, high and low values of ACTH concentration correspond with similar values in adrenal cholesterol concentration. On the 10th day of lactation pituitary ACTH concentration shows a slight increase whereas adrenal cholesterol concentration shows a further decrease (Fig. 1).

DISCUSSION

Total and ester cholesterol values, determined in this investigation, for the diestrous rat are in close agreement with those reported by others (4, 7, 25, 26). Results of this study indicate that following conception there is a gradual increase in adrenal cortical secretory activity which reaches a peak on the 15th day of pregnancy. Concentrations of total and ester cholesterol were found to be lower at this point than at any other time throughout pregnancy. The concentration of total cholesterol observed on the 15th day of pregnancy is in close agreement with the value reported by Longwell and Berenbeim (7) for the 16th day, and by Andersen and Sperry (4) for the 18th day. Pituitary ACTH determination as carried out in this investigation showed a decreased potency from diestrus to the fifteenth day of pregnancy.

Observations on adrenal cholesterol concentrations indicate that from the 15th day of pregnancy to the 21st day, adrenal cortical activity decreased. Parturition is accompanied by a sharp increase in adrenal cortical activity, as indicated by the rapid decrease in adrenal cholesterol observed between the 21st day of pregnancy and 1 hour following parturition. Longwell and Berenbeim (7) reported increases in adrenal cholesterol between the fifteenth and twenty-first day of pregnancy, while Andersen and Sperry (4) reported cholesterol values for the time of parturition that are similar to the ones observed in this study. Changes observed in pituitary ACTH potency complement the observation on adrenal cortical activity.

The fact that pituitary ACTH potency increased from the fifteenth to the 21st day of pregnancy is interpreted as evidence of decreased ACTH release and is congruous with the observed decrease of adrenal cortical activity. The sharp decrease in pituitary ACTH potency observed during parturition is compatable with the sharp increase in adrenal cortical activity noted during this same period.

Similar changes in adrenal cortical activity during pregnancy have been reported by Venning (5) and Schultze (10). Gemzell (6) reported an increase in the level of circulating 17-hydroxycorticosteroids at the beginning of the last trimester of pregnancy, but he did not observe any decrease from this time to the time of parturition. A possible explanation for the variation between the observations of Gemzell and those of this study is that in the latter investigation only the activity of the maternal adrenal gland was studied whereas Gemzell studied blood 17-hydroxycorticosteroids which may have had their origin in the maternal or fetal adrenal glands. There is evidence that in the rat the fetal adrenals contain lipid droplets at 16 days of gestation and may be actively secreting by the 19th day (27). Possibly the decreased activity of the maternal adrenal observed in this investigation during the last trimester of pregnancy, may be related to the increased activity of the fetal adrenals that occurs at this time.

The indication in this study that a sharp increase in the activity of the pituitary-adrenal axis occurs during parturition is in agreement with that previously recorded (Andersen and Sperry, (4); Shaw (13); Gemzell (6); and Schultze (10)). The physiological stress associated with the process of parturition has been suggested as an explanation for this increased activity. It also seems possible that estrogen may be a contributing factor in this increased activity. It is well known that urinary estrogen excretion rises rapidly during the late stages of pregnancy. Similar increases in serum estrogen have been reported (28). It has been shown that estrogens can cause a ten-fold increase in blood ACTH within 24 hours (29), an increased adrenal gland size (30), and a decrease in adrenal cholesterol (31).

Increased adrenal cortical activity associated with lactation has been reported by Bromberg, Feldman, and Eliakim (14), and Andersen and Sperry (4) have reported that by the twenty-second day of lactation adrenal cholesterol concentration was still somewhat lower than non-pregnant levels. It has been established, beyond reasonable doubt that a functional adrenal cortex is essential for normal lactation in the rat (32, 33). The increased activity of the adrenal cortex during lactation, as observed in this study, is congruous with the increased physiological demand imposed on the lactating animal by actively secreting mammary glands. There is evidence that in mice and rats mammary gland activity increases during the first two weeks of lactation and then tends to subside (34, 35, 36). In this study observations on adrenal cortical activity indicate that

concomitant changes in adrenal cortical activity and mammary gland activity occur in the rat.

Only slight regression in adrenal cortical activity during the latter half of lactation was observed. There is evidence in the rat that a sharp increase in the rate of protein deamination occurs during the latter half of lactation (37) for which a functional adrenal gland has been shown to be necessary (38). This evidence can be interpreted as an indication that in the mammary gland of the rat the 11-oxygenated corticosteroids may play a direct role in supplying milk sugar precursors from non-carbohydrate sources. This activity appears to be greatest during the latter half of lactation.

SUMMARY

The activity of the anterior pituitary-adrenal cortex axis has been studied in the laboratory rat during diestrus, pregnancy, and lactation. Adrenal cortical activity was assessed by measuring the concentration of total and free cholesterol in the adrenal gland. Pituitary ACTH potency was determined by the ascorbic acid depletion technique.

The concentration of total adrenal cholesterol was: 2.79 mg. per 100 mg. of adrenal tissue in the diestrous rat; 1.81 mg. on the 15th day of pregnancy; 2.79 mg. on the 21st day of pregnancy; 1.38 mg. one hour after parturition; and 0.81 mg. on the 10th day of lactation.

The milliunits of ACTH per 200 μg. of anterior pituitary tissue were: 0.90 in the diestrous rat; 0.77 on the 15th day of pregnancy; 1.20 on the 21st day of pregnancy; 0.73 one hour after parturition; and 0.95 on the 10th day of lactation.

The possible factors regulating the observed changes and activity of the pituitary-adrenal gland axis during pregnancy and lactation are discussed.

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STUDIES ON THE ADRENAL DURING SIMULTANEOUS ADMINISTRATION OF DDD AND AMPHENONE

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IN 1948 Nelson and Woodard (1, 2) reported that oral administration of DDD (2,2-bis(parachlorophenyl)-1,1,dichloroethane) an analog of the common insecticide DDT, causes atrophy of the adrenal cortex and further that this atrophy is species specific for the dog. Several papers have since appeared dealing with the alterations of physiology induced by this compound but no data concerning the mechanism of action has been recorded apart from the postulation by one of us, J.N. (3), that the substance might act by "blocking" the effect of ACTH. A few reports indicate that the compound may have an effect in species other than the dog; these will be discussed later. Hertz *et al.* (4) have established that Amphenone causes cessation of function of the adrenal and subsequent engorgement of the cortical cells with cholesterol and hypertrophy. This hypertrophy is due to the rise in plasma ACTH and does not obtain in the hypophysectomized animal. Amphenone is effective in all species of animals so far studied. It naturally follows that simultaneous administration of both compounds to dogs and to rats might yield data of interest. The following is a report of such an experiment.

MATERIALS AND METHODS

Because of the small supply of Amphenone and because puppies weigh less than adult dogs, thereby requiring less Amphenone,[3] it was considered best to carry out the dog experiments with litters of puppies of about two weeks of age. This had the further advantage of providing a somewhat homogenous test group, a situation difficult to obtain in the mongrel dog. The authors know of no important differences between the adult dog and pup adrenal. The first litter consisted of nine pups. Three were given 200 mg./Kg. of DDD[4] dissolved in corn oil daily by stomach tube, together with 200 mg./Kg. of Amphenone. The Amphenone was freshly dissolved in distilled water. All

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[3] Amphenone kindly supplied by the Upjohn Company, Kalamazoo, Michigan. The revised formula for amphenone is 1,1-bis(para-aminophenyl)-1-methyl propanone (34).

[4] The DDD used in this experiment is the technical product purchased on the open market and is of the same grade as that used in all previous experiments. The active principal is contained in the ether soluble mother liquor (35).

compounds were given in divided doses at 8-hour intervals by stomach tube. Three other pups were given the same dose schedule of Amphenone only without the DDD. The remaining group of three pups was given the same dose schedule of DDD only without the Amphenone.

The pups receiving Amphenone and DDD simultaneously all died after the third dose and within 24 hours after beginning the experiment. The pups receiving Amphenone only died after the fifth dose on the second day. The pups receiving DDD only all died on the seventh day.

The next litter of ten pups was divided into three groups. The first group of four received DDD 25 mg./Kg. daily together with Amphenone 100 mg./Kg. daily in three divided doses. The second group of four pups received Amphenone only in the same dose schedule as above without DDD while the third group of two pups were untreated and considered as controls. The first pup receiving simultaneous Amphenone and DDD died on the 10th day. On the 11th day the remaining nine were killed. During the time while the compounds were being administered the pups were nursed by their mother who received generous supplements of horse meat and milk. Further, each pup received 45 ml. of whole cow's milk per day by stomach tube in three divided doses.

The experiments on rats were conducted on 35 males of the Wistar strain which were purchased from a dealer. Ten rats were given orally 200 mg./Kg. of DDD in corn oil per day and 200 mg./Kg. Amphenone per day freshly prepared in distilled water and administered in three divided doses at 8-hour intervals. Ten rats were given the same dose schedule of Amphenone only and another group of 10 rats were given the same dose schedule of DDD only; the remaining 5 rats were treated as controls. Two rats from each of the three groups receiving both compounds were killed on the sixth day and the remaining 29 were killed on the fourteenth day.

The adrenals from all animals apart from the three pups dying from DDD on the seventh day were dissected free of fat, weighed, and the right adrenal fixed in formalin while the left gland was fixed in Zenker's solution. Frozen sections from the formalin fixed gland were stained with sudan III and the Schultz reaction for cholesterol was performed. Sections from Zenker fixed material were embedded in paraffin and stained with hematoxylin and eosin in the usual fashion.

RESULTS

The weights of the animals, treatment they received, and weights of the adrenals are noted in Table 1. Here it can be seen that the puppies on the 200 mg./Kg. dose schedule of Amphenone with and without DDD show essentially no weight change in the adrenals in the short time of the study. It is noted that the pups receiving both compounds die sooner than pups receiving either alone. It must be born in mind that there is a natural high mortality rate of normal newborn pups. However, these pups were two weeks of age and this litter was selected because the pups were uniformly robust. They had been given milk supplements several days prior to the start of the experiment. It is felt that this early death is a real additive effect of both compounds because the same effect has been noted in another series of adult dogs in a separate study.

In the case of puppies on 100 mg./Kg. of Amphenone it can be seen that the hypertrophy which occurs is of somewhat less magnitude than that induced by twice this dose in rats and further that this hypertrophy can be

TABLE 1

Initial weight	Treatment	Weight after seven days	Combined adrenal weight
Pup 1. 600 gm. Pup 2. 550 gm. Pup 3. 520 gm.	200 mg. DDD plus 200 mg. amphenone per Kg./body weight	———	75 mg. 68 mg. 66 mg.
Pup 4. 580 gm. Pup 5. 610 gm. Pup 6. 625 gm.	Amphenone only Amphenone only Amphenone only	——— ——— ———	60 mg. 72 mg. 73 mg.
Pup 7. 605 gm. Pup 8. 575 gm. Pup 9. 550 gm.	DDD only DDD only DDD only	615 gm. 600 gm. 590 gm.	Too small to identify with certainty
		Weight after 10 days	
*Pup 10. 1040 gm. Pup 11. 920 gm. Pup 12. 985 gm. Pup 13. 865 gm.	DDD 25 mg./Kg. plus 100 mg. amphenone per Kg.	1180 gm. 1115 gm. 1120 gm. 1005 gm.	28 mg. 25 mg. 34 mg. 30 mg.
Pup 14. 1100 gm. Pup 15. 1060 gm. Pup 16. 1005 gm. Pup 17. 1020 gm.	amphenone 100 mg. per Kg.	1070 gm. 1165 gm. 1205 gm. 1230 gm.	165 mg. 157 mg. 140 mg. 130 mg.
Pup 18. 975 gm. Pup 19. 990 gm.	Control Control	1400 gm. 1380 gm.	92 mg. 114 mg.

No. of rats	Treatment	Weight at start gm.	Weight when killed on 6th day gm.	Weight when killed on 14th day gm.	Weight of adrenals in mg on 6th day	Weight of adrenals in mg. on 14th day
10	DDD plus Amphenone	204 ± 6*	210 216	212 ± 10	70 mg. 85 mg.	104 ± 14
10	DDD only	202 ± 8	205 215	215 ± 11	62 mg. 54 mg.	63 ± 10
10	Amphenone only	205 ± 9	200 212	219 ± 12	73 mg. 78 mg.	109 ± 13
5	Controls	208 ± 5		244 ± 12		60 ± 12

$$* = \sqrt{\frac{\Sigma d^2}{N(N-1)}}$$

prevented by simultaneous administration of DDD. Administration of Amphenone does not prevent atrophy caused by DDD and this atrophy in the pup is as profound as atrophy induced in the adult dog by 7–8 times the dose of DDD. It can be seen that in the case of the rat Amphenone induced hypertrophy is not inhibited by simultaneous administration of DDD and further, DDD alone does not induce atrophy.

Histological examination of adrenals from the rats receiving Amphenone and DDD and Amphenone and which were killed on the sixth day reveals the organ to be hypertrophied. The cells of the zona fasciculata and zona reticularis are engorged with lipids in small and large droplets. These cells

stain poorly with hematoxylin and eosin, the cytoplasm and cell boundaries are indistinct and the nucleus is poorly differentiated from the cytoplasm. In some cases the nucleus stains eosinophilic. The cells of the zona glomerulosa appear to be increased in number and to be compressed. The cytoplasm is scanty and the nucleus is dense, being markedly basophilic (Fig. 1). Sudan stains reveal the inner zones to be engorged with lipids and the Schultz reaction for cholesterol is positive. The zona glomerulosa is devoid of lipids (Fig. 2). The adrenals of the rats killed on the 14th day show that these changes of the inner zones have extended to the zona glomerulosa

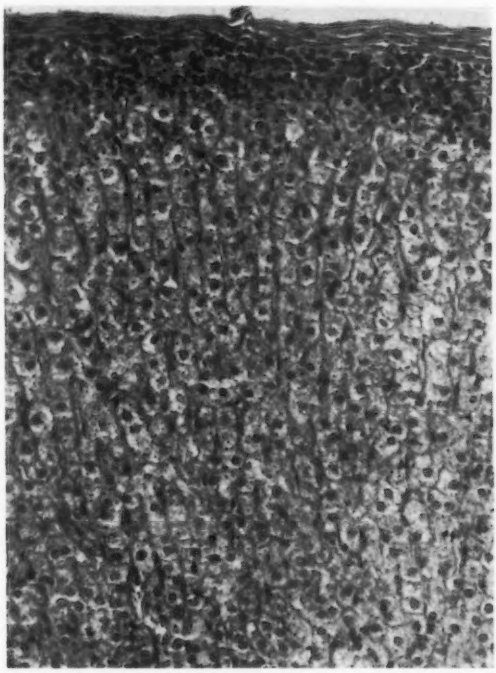

FIG. 1. Section of adrenal cortex from a rat receiving Amphenone and killed on the 6th day. Note the compact small cells of the zona glomerulosa with small basophilic nuclei and the lipid engorged cells of the zona fasciculata. H & E.

which is now also rich in lipids and cholesterol. This zone is almost indistinguishable from the inner zones. The adrenals from some of the animals show extensive areas of liquefactive necrosis with areas of polymorphonuclear leucocyte infiltration. The adrenals from the rats treated with DDD only show no changes from normal.

Sections of the adrenals from the pups treated with Amphenone only and dying on the 19th and 11th days show the cells of all zones to be distended with large and small globules of lipids. The cells stain poorly and are identical with the cells in the adrenals of the rats treated with Amphenone. The depletion of lipids from the zona glomerulosa was not noted, perhaps because the puppies were not killed early enough in the experi-

ment. In the puppies given DDD 25 mg./Kg. and Amphenone simultaneously none of the foregoing changes was noted. The findings were identical with those of adult dogs receiving 200 mg./Kg. DDD. The histological appearance of the dog adrenal treated with DDD has been adequately described elsewhere (5) and consists mainly of atrophy of the inner zones which are replaced by a band of condensed stroma leaving a relatively normal zona glomerulosa.

Microscopic examination of the adrenals from the pups dying on the second day with the 200 mg./Kg. dose of Amphenone and without DDD showed only mild infiltration of lipids in the zona fasciculata. The adrenals from the pups receiving DDD only at the 200 mg./Kg. dose and dying on the 7th day were markedly atrophic and difficult to find. These structures could not be identified with certainty until confirmed by histological examination. Figure 3 shows the remains of one such adrenal. It is characterized by a fibrous band, presumably the capsule, beneath which are a few poorly identified cells some containing large globules of lipid in the cytoplasm. A normal medulla is present.

DISCUSSION

In the original paper Nelson and Woodard reported DDD to be species specific for the dog. This has been the finding of most workers. However,

FIG. 2. Sudan stained frozen section of the same gland as fig. 1. Note absence of lipids in the zona glomerulosa.

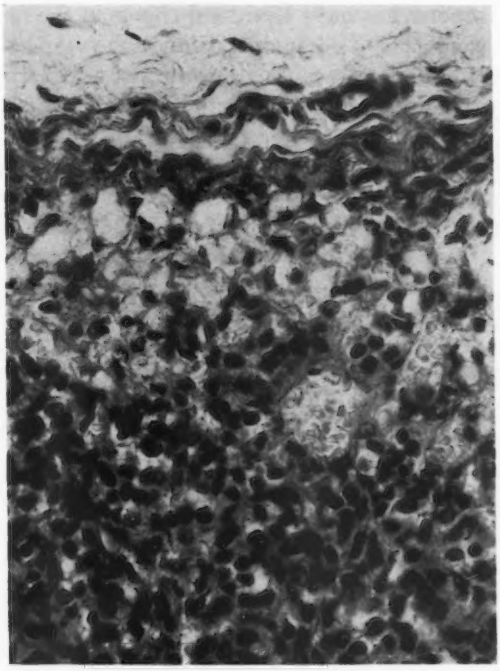

FIG. 3. Section of adrenal cortex of pup fed DDD and dying on the 6th day. The outermost layer is the capsule. The inner band of cells are unidentifiable and are all that is left of the cortex. The innermost layer of cells is the medulla. H & E.

Verne and Wegman (6) reported that DDD given parenterally causes atrophy of the rat's adrenal. These workers place considerable emphasis on the fact that the suspension was supersaturated and stabilized with Tween 80 (7). This has not been confirmed in the rat by Stoner (8) and in the mouse by D'Arcy (9). Brown (10, 11) has obtained amelioration of alloxan diabetes and sensitivity to cold in the rat which he considers adrenal hypofunction but he does not report histological atrophy of the adrenal, this has been confirmed in the rabbit (12). Other workers have reported the compound to have produced equivocal histological changes in the adrenal of the rabbit (13, 14). DDD was originally reported to be without anatomic or physiological effects in the human (15–16) although recently Zimmerman *et al.* (17) have reported regression of carcinoma of the breast which they consider an equivocal effect of the compound and Törnblom (18) reports partial success in the treatment of diabetes after prolonged therapy. All of the effect of lowering the blood sugar may not be mediated through the adrenal cortex because Candela and Candela (19) have shown that DDD increases the sensitivity of the isolated rat diaphragm to insulin.

Another compound known as Perthane (Q-137) which has ethyl groups substituted for the chlorine atoms on the benzene rings is reported also to cause histological atrophy of the dog adrenal (20). This compound is

reported to have equivocal effects in the mouse which are considered to be the result of adrenal hypofunction (21). Taliaferro *et al.* (22) have reported this analog to cause a lowering of the plasma steroids in a series of human cancer patients and further the usual rise in steroids elicited by ACTH does not occur after Perthane therapy.

No idea as to the mechanism of action of these compounds is at hand apart from the original suggestion that the DDD might block the effect of ACTH. This was derived from the finding that DDD accumulates in the body fat depots and is present in the adrenal. Since the zona glomerulosa contains lipids the substance could be considered present in this zone also. However, this zone does not atrophy at the 200 mg./Kg. dose in the adult dog but remains intact as does the zona glomerulosa of the dog after a comparable post-hypophysectomy interval. Certain other findings would seem to support this theory, thus, adrenal slices from DDD-treated dogs do not show the usual increase in O_2 uptake when ACTH is added to the menstrum in the Warburg apparatus (23) and the intact dog does not show the usual biochemical effects of ACTH administration after pretreatment with DDD (24). Also the Perthane fed animal does not show the usual rise in plasma 17-OH cortico steroids in response to ACTH (25). It must be noted that all of the foregoing experiments suffer from the defect that the adrenal is, at least partly, atrophic by the time the ACTH is administered. From the extent of atrophy and short time required to cause death in the pup, it would seem that DDD has an atrophying effect directly on the adrenal cortical cells in addition to any ACTH blocking effect. This increased sensitivity of the pup adrenal to DDD has been confirmed in a separate litter of five. The early death of the pup could be considered partly due to adrenocortical insufficiency especially since Larson *et al.* (20) have shown that in the adult dog treated with Perthane the life can be prolonged by cortisone therapy.

The adrenal hypertrophying effect of Amphenone is not species specific and is probably due to a rise in plasma ACTH. Rosenfeld and Bascom (27) have shown that in calf adrenals perfused with Amphenone there is an inhibition of biogenesis but not of release of the steroid hormones. This lowering of plasma steroids would enhance the output of ACTH and thereby cause the hypertrophy of the inner zones of the gland which does not occur in the hypophysectomized animal (4). The foregoing concept must be re-evaluated in view of the findings of Vogt (28) who reports that intravenous Amphenone in a short term experiment causes a low output of cortical hormones in the adrenal vein of the rat while orally administered Amphenone over a two week period causes an increased output of cortical hormones and this increased output is parallel with the hypertrophy of the gland. She concludes that the action of Amphenone is complex and that it probably acts on the pituitary in addition to the adrenal.

The finding that Amphenone causes hypertrophy of the rat's adrenal while DDD is simultaneously being administered would seem to confirm that DDD is without effect in the rat, either as to a direct atrophying effect or the blocking of ACTH. The finding that DDD prevents hypertrophy of the adrenals in the dog fed Amphenone would seem to support the suggestion that DDD acts by blocking the effect of ACTH in this species. The direct toxic effect of DDD on the adrenal cells of the dog may act in such a fashion that the enzyme systems responsible for maintenance of the cell are destroyed and any steriodogenic enzyme systems of such a cell would be refractory to ACTH.

From the histological observations of deposition of lipids in the zona fasciculata and depletion of lipids in the zona glomerulosa it is reasonable to presume that Amphenone has caused cessation of hormone formation in these inner zones. These inner zones may show these changes partly as a result of increased plasma levels of ACTH as well as any direct effect of Amphenone on the cells themselves. These histological changes closely parallel the known dependence of the inner zones on ACTH. The finding that the zona glomerulosa is relatively normal in the rats during early administration with Amphenone would suggest that this zone is continuing to function. Evidence of electrolyte impairment has not been observed in the rat (4). Early reports on the effect of Amphenone in the human indicated that impairment of electrolyte balance is a late phenomena (29); however, a later report (30) indicates small doses of Amphenone to cause a decreased secretion of aldosterone. Depletion of lipids from the zona glomerulosa occurs in Na-K imbalance and has been interpreted as indicating this zone controls electrolyte metabolism in the rat (31, 32) and in man (33). After continued Amphenone administration the zona glomerulosa undergoes the same changes as does the zona fasciculata which progresses to necrosis.

SUMMARY

Simultaneous administration of DDD and Amphenone to dogs and rats reveals that the atrophy of the dog's adrenal produced by DDD is not influenced by Amphenone. Amphenone produces hypertrophy in the adrenal of both the dog and rat and this hypertrophy is prevented by simultaneous administration of DDD in the dog but not in the rat. The hypertrophy is associated first with the inner zones of the cortex and consists of swelling of the cells, engorgement with lipids, disruption, loss of normal staining characteristics and lastly necrosis. The pup is more sensitive than is the adult dog to the adrenal atrophying effect of DDD. The fact that the zona glomerulosa can be destroyed with DDD in the pup suggests that this substance has a direct effect on the cortical cells in addition to any effect which blocks the action of ACTH.

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THE EFFECT OF NATURAL AND SYNTHETIC ESTROGENS ON RETICULOENDOTHELIAL SYSTEM FUNCTION[1]

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RECENT experiments in this laboratory (1); and by Halpern, Benacerraf, and Biozzi (2); Friedman and Byers *et al.* (3, 4, 5); Rice *et al.* (6); Brown *et al.* (7); and Lautsch (8) have indicated that the reticuloendothelial system (RES) plays an important role in cholesterol metabolism.

In general, these data show that cholesterol-containing macromolecular aggregates ranging in size up to chylomicrons appear to be phagocytosed by RE cells. Blockade of the RES will cause a significant increase in the time that it normally takes for an induced hypercholesterolemia to disappear. On the other hand, stimulation of the RES (9) will accelerate the reduction of blood cholesterol levels.

While work was being done on the role of the RES and cholesterol, other studies by Oliver and Boyd (10, 11, 12) and Barr *et al.* (13, 14, 15) were being carried out on the effect of certain estrogens in experimental and clinical atherosclerosis as well as xanthomatosis. These studies, predicated on the observation that pre-menopausal women are virtually immune from coronary thrombosis, indicate that certain estrogens can cause a clinical improvement as well as a reversion toward the norm of the blood lipid picture in hypercholesterolemic individuals. Using histologic techniques, Nicol and his associates (16, 17, 18, 19) indicated that estrogenic substances had a stimulatory effect on the RES.

Thus, there are indications that the estrogens may have a useful role in therapy or prophylaxis of disorders associated with abnormal concentrations of cholesterol and that the efficacy of these substances may be due to their effect upon the RES. A major limiting factor in the clinical use of such steroids lies in their concomitant sexual effect. Hence, it was felt that a useful purpose could be served by attempting to determine whether steroid structure might be altered so as to reduce sexual side effects to negligible values but still maintain the RES stimulating effect. This is a report of an investigation into this problem.

METHODS

In determining RES activity, the test animals used were twenty-gram male, white Swiss mice. The testing agent was colloidal carbon. Carbon particles with a mean size of

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19 mμ, as determined by electron microscopy, were suspended in a 4% gelatin solution with a concentration of 21.3 mg. of carbon per cc. One cc./100 gm. mouse was injected intravenously. Negatively charged colloidal particles of appropriate size are selectively and exclusively phagocytosed from the circulating blood by RE cells, as can be seen in the micrographs and microradioautographs in Figures 1–6. Histological evaluation of

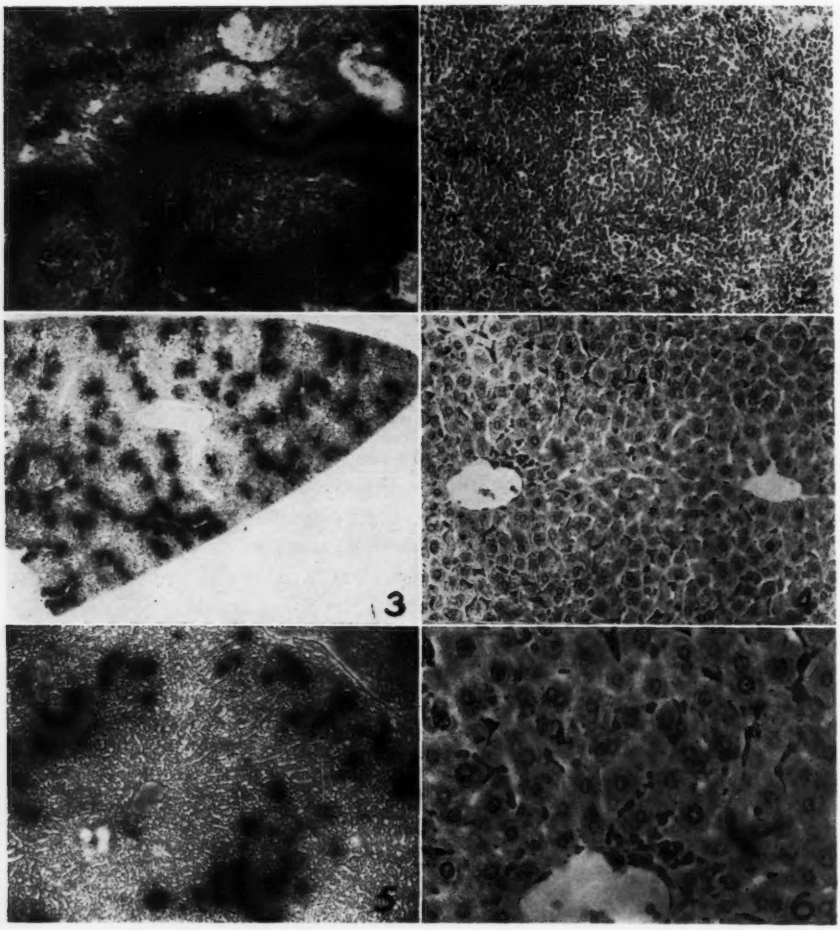

FIG. 1. Radioautograph of the spleen showing localization of $CrP^{32}O_4$ in RE cells.

FIG. 2. Histologic section of the spleen showing phagocytosed carbon in RE cells.

FIG. 3. Radioautograph of liver showing the perilobular disposition of $CrP^{32}O_4$.

FIG. 4. Histologic section of liver showing carbon in Kupffer cells in a perilobular distribution.

FIG. 5. Enlarged radioautograph of figure 3 showing specific localization of radioactive chromic phosphate in the Kupffer cells.

FIG. 6. Enlargement of figure 4 showing the heavy concentrations of carbon in RE cells.

all the tissues of the body including blood smears demonstrates that none of these colloids is taken up acutely by any cells of the body other than those of the RES. Samples of blood containing 10 λ each were drawn from the tip of the tail at five minute intervals. The blood was put in 1.2 cc.'s of 0.01 molar sodium carbonate and read in a colorimeter against a blood control at a wave length of 6000 Å. The blood carbon concentration was plotted semilogarithmically against time. Twelve animals were used for each point. No data were used unless the P value was <0.01.

The steroids were synthesized by Dr. Max Huffman and dissolved in absolute ethanol. Sesame oil was added and the alcohol evaporated as far as was feasible. The final concentration of steroid was 10 mg/1 cc. One mg. of each steroid was injected subcutaneously each day for either 1, 2, or 4 days, depending on the experiment. Sesame oil controls were run against each steroid tested.

In order to measure radioactive colloid uptake in various organs, $CrP^{32}O_4$ was prepared as previously described (20). A known mass (which ranged up to 10 mg./20 gm. mouse) of radiocolloid containing 200 μc. of P^{32}/10 mg. of $CrP^{32}O_4$ was injected into the tail vein of each mouse. One hour later the animals were sacrificed and liver, spleen, lung, kidney, heart, femur, and some striated muscle were immediately removed, weighed, and ashed in porcelain crucibles in 500° F for 3 hours. The assayed radioactivity was calculated as per cent of the injected dose, radioactivity per gram, and total radioactivity of the organ. Peripheral blood determinations were made from tail-tip blood, and bone marrow examinations were made from femoral marrow.

RESULTS AND DISCUSSION

In all cases, sexually active estrogenic substances, both natural and synthetic, had a powerful stimulatory effect on the phagocytic velocity of the RES. Figure 7 shows the normal disappearance velocity of carbon with the standard deviation. The effect of estrogens can be observed in Figure 8.

In addition to estriol and Δ1,3,5 estratriene 3, 16β-diol shown in Figure 8, other estrogens including stilbestrol, ethynyl estradiol, and estrone were tested and all proved to be very active RES stimulants.

The search for steroids whose effect on the RES would be maintained but whose sexual effect was less was begun with the isomer of estradiol. Experimentation was confined primarily to groups on the 3, 16, and 17 positions. Table 1 lists those synthetic substances that were active on the RES as well as those that were inactive. The criterion for activity was a minimum of 100% increase in phagocytic velocity. Increments of more than 400% were common. In reviewing those elements of structure which appear to be useful in stimulation of the RES, it is apparent that a Δ1,3,5 estratriene structure with oxygens in the 3, 16, and 17 positions are active. If the oxygen in the 17 position is removed, the molecule will maintain its activity if there is a β hydroxy or a keto group in the 16 position. Hence, Δ1,3,5 estratriene 3, 16 β-diol is active as an RES stimulant but has only about 1/1000 of the sexual activity of estradiol. If the hydroxy group in the 16 position is changed so that there is a 3, 16 α-diol, most RES activity is lost. In the case of the 16 keto group, the introduction of a methoxy group on the 3 position instead of the hydroxyl group also leads to a loss of almost all activity. In other structures such as 16 epiestriol, a methoxy or ethoxy

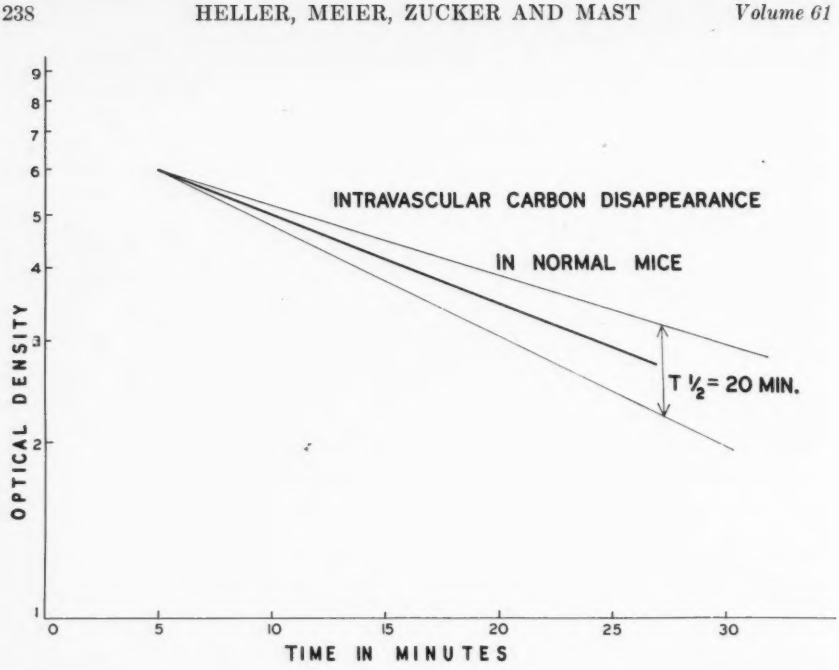

FIG. 7. Exponential disappearance of intravascular, electro-negatively charged colloids in mice. The lines demarcated by the double-headed arrow indicate the standard deviation.

group on the 3 position increased RES activity. It will be noted that none of the androgens tested have any activity at all.

After the RES determinations were made, the animals were sacrificed and autopsied. In all cases the livers and spleens were weighed. A universal finding was a statistically significant increase in the size of the liver and spleen in those animals where increased RES activity had been observed. It was logical to assume that since this increase in size paralleled the increase in phagocytic velocity that the increment in mass would be due to proliferation of RE elements. Since the steroids had been given over a 4

TABLE 1. ACTIVE AND INACTIVE STEROIDS

Active	Inactive
estradiol	Δ5 androstene 3β, 16β-diol
ethynyl estradiol	androstane 3μ, 16β-diol
estradiol 16-one	androstan 3α-ol, 16-one
Δ1,3,5 estratriene 3, 16β-diol	Δ4 androstene 3, 16-dione
3 methoxy Δ1,3,5 estratriene 16β-ol	Δ5 androsten 3β-ol, 16-one
Δ1,3,5 estratriene 3-ol, 16-one	3β methoxy Δ5 androsten 16-one
estriol	Δ1,3,5 estratriene 3, 16α-diol
16-epiestriol	3 methoxy Δ1,3,5 estratrien 16-one
3 methoxy Δ1,3,5 estratriene 16β, 17β-diol	
3 ethoxy Δ1,3,5 estratriene 16β, 17β-diol	

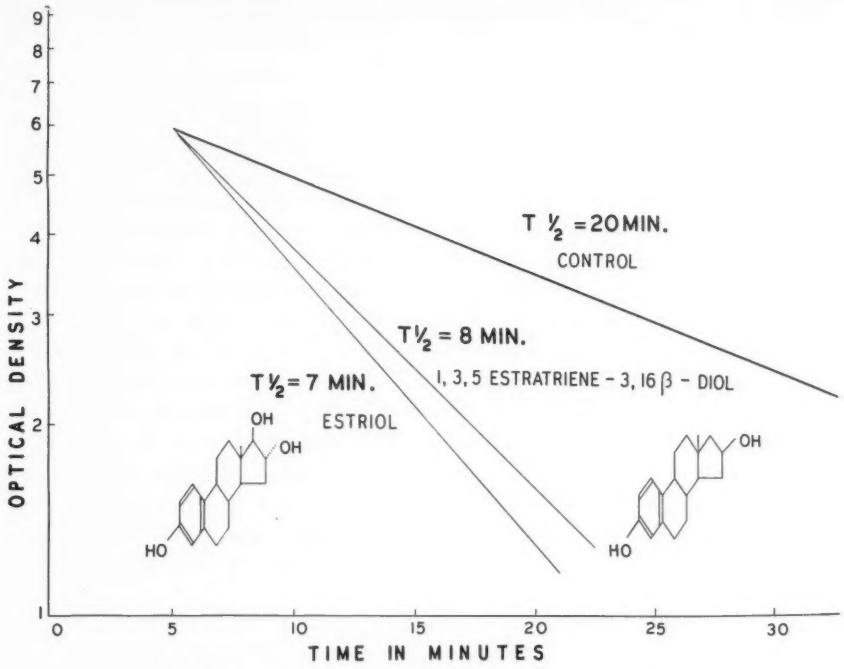

FIG. 8. Curves of intravascular carbon disappearance in control and steroid-injected mice.

day period, adequate time for such hyperplasia was allowed. In order to investigate whether or not this hypothesis was correct, a known mass of radioactive colloidal chromium phosphate was injected into animals that had received RES stimulatory steroid therapy. Had there been RES hyperplasia, the introduction of an adequate amount of radiocolloid into the vascular bed should have resulted in an increased uptake of colloid, particularly in the liver and spleen. We would anticipate, therefore, that the radioactivity per organ would be considerably greater, whereas the radioactivity per gram of organ would tend to remain the same. This was not the case at all. As can be seen from Table 2, there is a marked enlargement

TABLE 2. THE MEAN RADIOACTIVE UPTAKE OF NORMAL AND STEROID* INJECTED ANIMALS

	Wt. of animal	Wt. of liver	Liver RA per gram, cpm.	Total liver RA, cpm.	% in liver of total RA	Wt. of spleen	Total spleen RA, cpm.	Spleen RA per gram, cpm.	% in spleen of total RA
Control†	26.9	1,4061‡	48,034‡	67,288	64.2	0.1177‡	2,097‡	18,576‡	1.95‡
Exper.†	26.0	2,0327‡	35,453‡	69,321	66.2	0.2124‡	950‡	4,108‡	0.90‡

* P value <.01.
† 3 methoxy Δ1,3,5 estratriene 16β, 17β-diol (1 mg./day ×4).
‡ 12 animals in each group.

of the liver and spleen. The total radioactivity of the liver is increased very slightly, whereas the radioactivity per gram is markedly diminished. In the spleen, despite its twofold increase in size, the total radioactivity is markedly reduced and the radioactivity per gram is greatly reduced. These steroids have, in effect, depressed the absolute colloid uptake of this organ.

It was, of course, remotely possible that other organs in the body might be responsible for a markedly accelerated uptake of radiocolloid due to proliferation of RE cells, and, hence, other organs were subjected to scrutiny. Examination showed that there was no significant difference in the radioactive uptake by the RES in control and experimental animals where lung, kidney, striated muscle, heart, and femur were assayed.

Peripheral blood and bone marrow smears were made in all experimental groups. No significant difference between experimental and control groups was observed. Since all intravenously injected colloid can be shown to go exclusively to the RES, it is obvious that if an increased phagocytic velocity cannot be explained by the presence of new cells, then the explanation must lie in each cell phagocytosing more effectively and rapidly. Since the experiments with radiocolloids indicate that there is no absolute increase in RE elements, one is compelled to assume that the efficiency of the RE cells is markedly increased.

One may further assume that the increase in phagocytic velocity after injection of active steroids would, in all probability, be due to surface membrane phenomenon in the RE cell. It was assumed that if the steroid effect was, indeed, a cell membrane action, it would be unnecessary to give the steroids for 4 days. Thus, if one assumes that the steroid is taken up with reasonable speed from the subcutaneous depot and if the effect is upon the RES cell membrane, then an increased phagocytic velocity should be demonstrable within a matter of hours. One mg. of 16-epiestriol was injected subcutaneously into 12 mice. Twelve hours later the phagocytic velocity was increased 100 per cent over the control group. Although the increase phagocytic velocity was not as great 12 hours after subcutaneous injection of steroid as it was after 4 days of therapy, the effect is quite distinct. The reason for the increase in weight of the liver and spleen is still unexplained and will be the subject for further investigation.

SUMMARY

The RES stimulatory effect of a variety of estrogenic substances was assayed.

Synthesized steroids were assayed which had markedly reduced sexual activity but which maintained their stimulatory activity upon the RES.

Certain conclusions were drawn as to the relationship of structure and RES stimulatory effect.

Splenomegaly and hepatomegaly were concomitant features of increased

RES activity. However, this increase in size was apparently not due to hyperplasia of RE elements.

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A METHOD FOR THE SEPARATION OF FOUR PHENOLIC ESTROGENS IN HUMAN URINE BY PAPER CHROMATOGRAPHY AND AN EVALUATION OF BIOLOGICAL ASSAY OF THESE COMPOUNDS

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THIS paper presents a method for the estimation of individual urinary estrogens involving enzymatic hydrolysis of conjugates, ether extraction, paper chromatographic separation and purification of estrone, estradiol-3,17β, estriol and 16-*epi*estriol and finally quantitative estimation of these compounds by bioassay. The method is suitable for the measurement of very small amounts of urinary estrogens such as are excreted in the urine of patients with mammary cancer. The specificity and sensitivity of estrogen bioassay will be examined in this communication.

Estrogens are known to be significantly involved in the genesis of mammary neoplasms in animals (1) and these ovarian hormones are sustaining factors of human breast cancer (2). A study of estrogen excretion and metabolism in women with breast cancer may further advance our understanding of the endocrine factors in the growth of these neoplasms and the mechanism of regression of some mammary cancers following endocrine ablative surgery.

Attempts have been made in the recent years by several investigators to develop suitable methods with sufficient sensitivity for the determination of the microquantities of individual estrogens present in human urine. Brown (3) and Bauld (4) succeeded independently in the separation and determination of estrone, estradiol-17β and estriol in human urine by column chromatography and colorimetric estimation of these compounds by the Kober reaction. These methods are effective for the estimation of relatively high levels of urinary estrogens in premenopausal and in pregnant women. In postmenopausal and in ovariectomized women the levels of estrogen excreted are so low that more sensitive and specific methods of estimation are required.

The excretion of total estrogens in postmenopausal women and in women who have undergone ovariectomy and adrenalectomy has been investigated (5). The limitations in comparing total estrogen excretion in different urine extracts are (1) the potential antagonism of these compounds in such bioassay. Hisaw, Velardo and Goolsby (6) showed that

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estriol reduced the effectiveness of estrone or estradiol in causing uterine growth when these steroids were administered together. Huggins and Jensen (7) showed that estriol and 16-*epi*estriol were able to inhibit to a limited extent the uterine growth induced by estrone administered concurrently, and (2) that the same uterine weight response can result from mixtures of different estrogens varying widely in composition.

MATERIALS AND METHODS

Materials: All the solvents were redistilled c.p. reagents. Estrone obtained from Dr. H. J. Ringold, Syntex, was purified and recrystallized and had a m.p. of 261–261.5° (corr.). Estradiol-17β obtained from the same source had a m.p. of 177.5–178.5° (corr.). Estriol obtained from E. A. Sharp of Parke, Davis & Co. had a m.p. of 285.5–286.5 (corr.) 16-*epi*estriol obtained from D. M. M. Huffman, Oklahoma Institute for Medical Research, had a m.p. of 290.5–291° (corr.)

n-heptane (Phillips Petroleum Co.) was treated with concentrated H_2SO_4 (2–3 changes), then washed with water and alkaline $KMNO_4$. Finally, it was distilled through a Podbielniak column (b.p. 97–98°).

Whatman paper No. 43 was washed by extraction with methanol in a Soxhlet type apparatus for 2 to 3 days. This is essential because of the presence of considerable amounts of estrogenic materials in this filter paper as determined by bioassay. The eluate from a 3×9 cm. strip of Whatman No. 43 paper contained an amount of estrogenic material equivalent to 0.04 μg. of estrone by bioassay. Upon extraction of the paper with methanol this estrogen was removed completely.

Hydrolysis of urinary steroid conjugates and ether extraction: The hydrolysis of estrogen glucuronides in human urine was carried out by enzymatic methods. An aliquot of 24 hours urine was acidified to pH 4.5. The aliquot used varied. In pre-menopausal and in pregnant women 100 to 300 ml. was used, whereas a 500 ml. aliquot was used in post-menopausal or castrated women. Five ml. of 1.0 *M* sodium acetate buffer (pH 4.5) was added to each 100 ml. of urine. β-Glucuronidase, 300 units (8) per 1 ml. of urine was then added. The urine was incubated at 30° C. for 48 hours. More prolonged incubation did not increase the yield.

Ether was used for the extraction of estrogens. The ether extract from the hydrolyzed urine was extracted with 20 ml. of 2 N NaOH four times which removed all estrogens into the NaOH extract from the ether. The NaOH extract was immediately acidified with concentrated H_2SO_4 to pH 1.5 to 2.0 and then reextracted with ether. The final ether extract was washed with $NaHCO_3$ and water and dried with Na_2SO_4 and evaporated to dryness.[2] All evaporations were carried out in a rotary evaporator *in vacuo* at a temperature below 45°. Small volumes of organic solvents were removed in a stream of nitrogen. The residue was dissolved in 1 to 2 ml. methanol prior to paper chromatography.

Separation and purification of estrogens by paper chromatography: The following method of paper chromatography gave a quick and simple separation, and purification of four estrogenic compounds in human urine.

(a) Preparation of the chromatographic tanks: All tanks were set up in a room with constant temperature of 32 ± 1° as described by Bush (9) and all solvents were equilibrated at this temperature.

[2] Although washing with $NaHCO_3$ is useful in removing some pigments from the urine extract, we have found that it also removes amounts of estriol and 16-*epi*estriol. Since our final determination of estrogens is by bioassay the presence of pigments will not interfere with the accuracy of the results. Washing with $NaHCO_3$ has now been eliminated from the extraction procedures.

(b) Solvent system: Two chromatographic systems were employed. The first step consisted of a separation of estriol and 16-*epi*estriol from the estradiol-17β and estrone. The mobile and stationary phases in this system were respectively the upper and lower layers obtained from the equilibration of n-heptane, 1000 ml. and methanol, 1000 ml. The separation between estradiol-17β and estrone was very satisfactory in this system since the distance between these two spots was wide. The estriol and 16-*epi*estriol regions were eluted from the first chromatogram and rerun in a second chromatography using Bush B_5 system (9).

(c) Preparation and running of the chromatograms: The position of the estrogens on the paper was determined by means of parallel runs with small amounts of pure estrogens. The paper was prepared as follows: it was divided into three strips, two narrow strips of 3 cm. in width on each side and a middle strip of 9 cm. in width. Mixtures of estrogens (15 μg. each) were applied as single spots on the outer strips, and the urine extract to be chromatographed was applied in four spots at a distance of 2 cm. from each other in the middle strip. All spots were applied along a line 12 cm. from one end of the paper. The urine extract was dissolved in 1 to 2 ml. of methanol and no more than 0.25 ml. was applied on each spot. The amount of methanol used for dissolving the urine extract was entirely dependent on the anticipated amount of estrogens present.

The runs were started by pouring the mobile phase into a trough by means of a funnel through a hole in the lid of the tank. With a 32 to 36 hour run for heptane: methanol system and 6 to 7 hours run for Bush B_5 system, the spots were well distributed over the paper and widely separated (Fig. 1a and 1b).

(d) Detection and elution of estrogens: After the chromatogram was removed from the tank, it was dried in air. The two outside strips containing the pure estrogens were cut from the paper and were sprayed with a mixture of equal volumes of 1% solutions of $FeCl_3$ and $K_3Fe(CN)_6$ in water as suggested by Meyer (10). This freshly prepared mixture reacts with phenolic estrogens to produce a bright blue color—Turnbull blue. Immediately after spraying the paper was washed in diluted HCl (10% v/v) and finally rinsed with water and allowed to dry in air. The spots on the paper are permanent. The middle strip is left unsprayed. A close correspondence between the positions on the chromatograms of the standard estrogens and estrogenic activity in the urine extract has been repeatedly demonstrated by bioassay. (See below.) The middle strip, 9 cm. wide$\times$45 cm. long, was then cut into 15 small strips of 3 cm. each beginning from $\frac{1}{2}$ cm. above the origin. Each strip was eluted with methanol in a specially made elution chamber in which small scale chromatography of many strips could be carried out (Fig. 2). The amount of eluate collected was about 5 to 8 ml. The eluates from strips corresponding to each standard estrogen were combined and evaporated to dryness in a stream of nitrogen. The eluates from intermediate strips which were believed to contain no estrogen were evaporated each separately to dryness. The residues were then dissolved in 4.5 ml. of sesame oil for bioassay.

Bioassay procedure: Bioassays were carried out according to the procedure of Evans, Varney and Koch (11) using immature female albino mice (strain CF_1) weighing 6.5 and 8.0 gm. (approximately 20 to 21 days old) obtained from Carworth Farms, Inc., New City, New York. The animals were maintained on an estrogen free diet (7) at about 75° with drinking water *ad libitum*. Each mouse received a total of 0.6 ml. of the extract in sesame oil in six doses. Standard dose-response curves for each estrogen were carried out in parallel with each bioassay.

The sensitivity and relative consistency of the uterine weight responses in immature albino mice makes the assay method of Evans, Varney and Koch very reliable providing that the extracts contain no other hormones than phenolic estrogens. This problem is eliminated by the use of specific methods of extraction and paper chromatographic purification of the steroids.

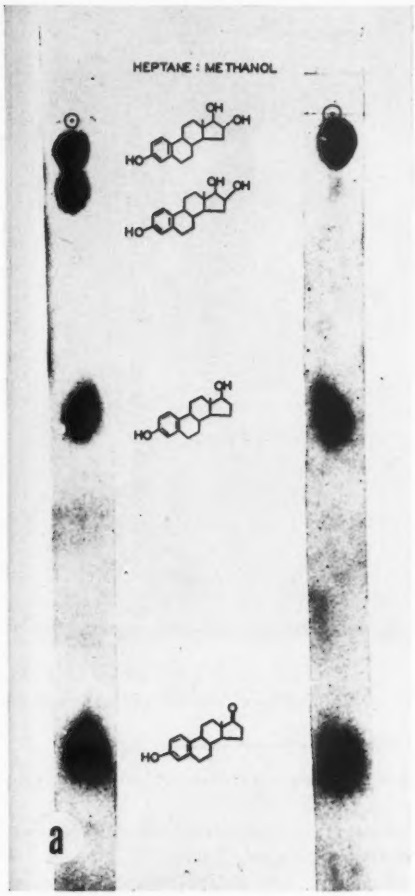

FIG. 1a. Separation of estradiol-17β and estrone from estriol and 16-*epi*estriol. Note the wide separation of estradiol-17β and estrone.

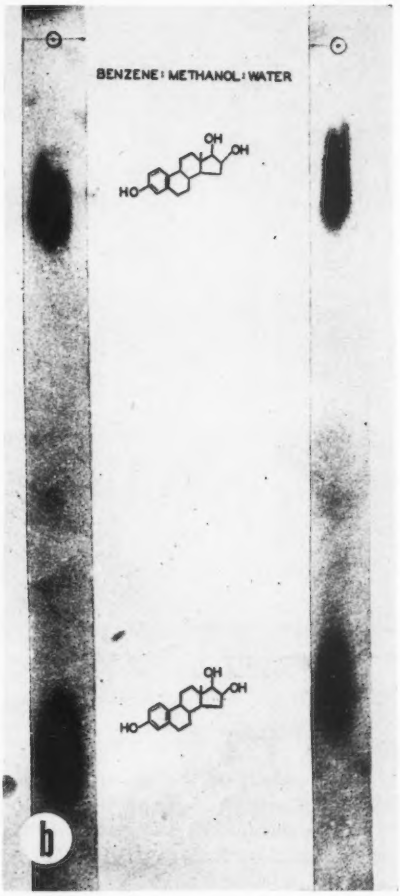

FIG. 1b. Separation of estriol and 16-*epi*estriol by Bush B_5 system.

Further appraisal of this method has been made on the results of our experiments and this enables us to conclude that this assay method can estimate very small amounts of each of four known estrogenic compounds in an aliquot of a 24-hour human urine specimen with reliability. Tables 1 and 2 illustrate that the standard deviations for all four estrogens assayed were low; in a series of 12 experiments the magnitudes of the standard deviations were a constant fraction of the uterine weight increase and amounted to $\pm 10\%$ of this increase. This indicates that in a 500 ml. aliquot of urine, a significant bioassay can be obtained for as little as 0.02 μg. of estrone; 0.005 μg. of estradiol-17β; 0.03 μg. of estriol and 0.06 μg. of 16-*epi*estriol. The uterine weight responses for the four estrogens at these dosage levels were compared with the uterine weights of the control animals in a series of 12 experiments and the differences were found statistically significant by the "t" test in all experiments ($p < 0.01$).

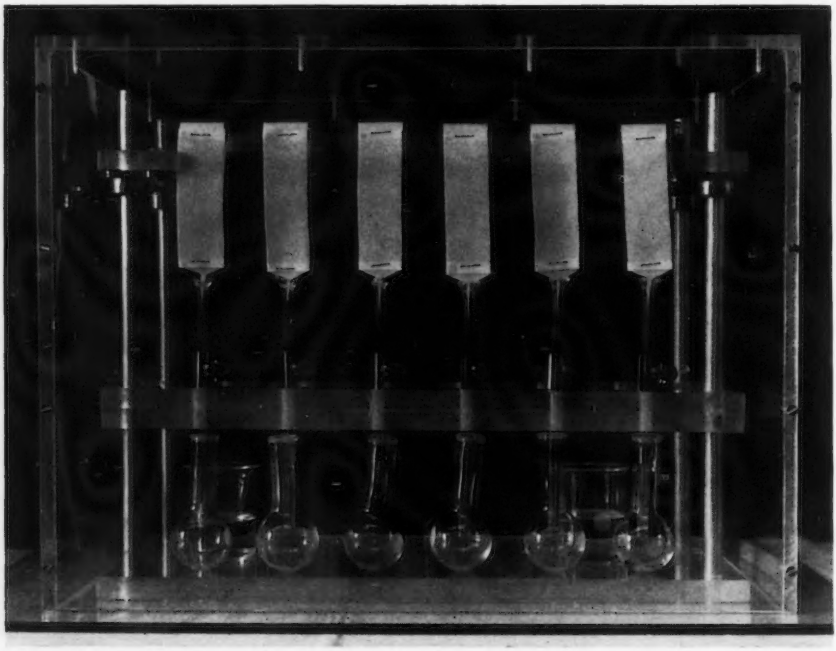

FIG. 2. Elution chamber: The middle strip was cut into many small strips of 3 cm. each and eluted by small scale chromatography in the elution chamber. 12 strips can be eluted simultaneously.

The accuracy of the bioassay depends on a significant increase of uterine weight which falls within the desired range of the dose-response curve.

The ranges for the best results for the bioassay of various estrogens are as follows: estrone, 0.02–0.12 μg.; estradiol-17β, 0.005–0.03 μg.; estriol, 0.03–0.09 μg. and 16-*epi*estriol, 0.06–0.09 μg. (Fig. 3). For the biologically very active estrogens such as estrone and estradiol-17β, bioassay of these compounds is satisfactory. The effective assay ranges for two estriol compounds, the impeded estrogens (7), are however narrow because the curve plateaus at much lower increase in uterine weight and the response to these weak estrogens is less sensitive (a three- to four-fold increase in doses produced only a ±30% increase of uterine weight). The assay of these compounds is

TABLE 1. UTERINE RESPONSE TO ESTRONE AND ESTRADIOL-17β

Estrone					Estradiol-17β				
No. of mice	Total dose, μg.	Mean uterine wt., mg.	Std. dev. ±	P value	No. of mice	Total dose, μg.	Mean uterine wt., mg.	Std. dev. ±	P value
6	Control	5.9	0.5		6	Control	5.9	0.5	
6	0.03	9.8	0.6	<0.01	6	0.005	9.5	0.4	<0.01
6	0.06	14.4	1.5		6	0.01	13.8	1.5	
6	0.12	21.1	3.2		6	0.03	24.4	1.9	
6	0.18	26.4	1.7		6	0.04	31.3	1.4	

TABLE 2. UTERINE RESPONSE TO ESTRIOL AND 16-*EPI*ESTRIOL

Estriol					16-*Epi*estriol				
No. of mice	Total dose μg.	Mean uterine wt. mg.	Std. dev. ±	P value	No. of mice	Total dose, μg.	Mean uterine wt., mg.	Std. dev. ±	P value
6	Control	5.2	0.5		6	Control	5.2	0.5	
6	0.03	7.9	0.3	<0.01	6	0.06	7.7	0.6	<0.01
6	0.06	9.6	0.3		6	0.09	8.8	0.8	
6	0.09	10.6	0.7		6	0.12	10.4	0.8	
6	0.12	12.1	0.3		6	0.18	11.2	0.3	

therefore less reliable, but significant assay can still be obtained if the amount to be assayed falls within the desired range. Under such circumstances multiple level assay is most desirable. Sample analyses of the assay of unknown urine extracts are shown in Tables 3 and 4.

Furthermore, if bioassay of estrogens is to be practiced with quantitative accuracy and reliability, the test animal should respond with minimum variability to the standard estrogens.

The relative potency of the four estrogenic compounds shows a remarkable constancy in a series of experiments (Table 5) extending over a period of 5 months. Such a lack of variation is indicative of the reliability and reproducibility of this assay method.

RESULTS

Paper chromatographic separation of human urinary estrogens: Studies were made on seven women: three normal premenopausal, one normal postmenopausal, one woman with 6 months pregnancy and two with car-

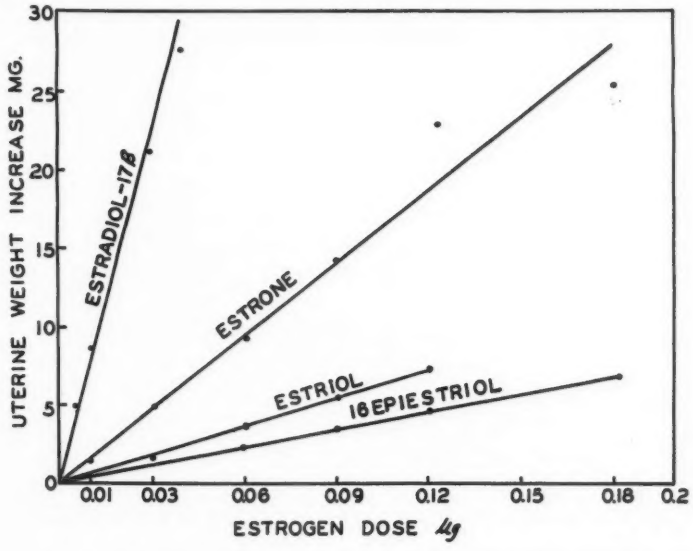

FIG. 3. Dose-response curves for 4 phenolic estrogens by bioassay.

TABLE 3. SAMPLE ANALYSIS

(1) 57-year old female, postmenopausal 10 years
Aliquot used for extraction = 500 cc.
The ether extract dissolved in 1 ml. CH_3OH. 0.8 ml. was applied on paper for chromatography.
Eluate dissolved in 4.5 ml. of sesame oil (each mouse receives 0.6 ml. of the total extract in oil)

	Uterine wt., mg. (mean)	Std. dev. ±	Increase uterine wt., mg.	Calculations	
				μg.	μg./24 hours
Estradiol-17β	18.3	1.6	12.6	0.15	0.3
Estrone	11.3	1.0	5.6	0.42	0.88
Estriol	5.8	1.0	0	0	<0.03*
16-*Epi*estriol	5.3	0.7	0	0	<0.06*

* Amount of these estrogenic compounds in the urine extracts was not large enough to cause any uterine weight increase.

TABLE 4. SAMPLE ANALYSIS

(2) 25-year old female, normal, 7th day of cycle
Aliquot used for extraction = 300 cc.
The ether extract dissolved in 2 ml. of CH_3OH of which 1 ml. was applied on paper for chromatography
Eluates dissolved in 4.5 ml. of sesame oil (each mouse receives 0.6 ml. of the total extract in oil)

	Uterine wt., mg. (mean)	Std. dev. ±	Increase uterine wt., mg.	Calculations	
				μg.	μg./24 hours
Estradiol-17β	12.1	1.7	6.2	0.012	0.7
Estrone	12.9	1.8	6.5	0.06	3.5
Estriol	10.2	1.5	4.3	0.078	6.5
16-*Epi*estriol	9.7	1.3	3.8	0.093	7.2

cinoma of the breast. Table 6 shows the results of the urinary excretion of estrogens in women without cancer. In pregnancy, the excretion of all estrogens was greatly increased. It was interesting to note that in a postmenopausal woman, excretion of estrone and estradiol was reduced considerably and epiestriol and 16-*epi*estriol were undetectable. Two women with metastatic carcinoma of the breast were studied for a long period of time prior to and following the removal of their endocrine glands. Varia-

TABLE 5. THE RELATIVE POTENCY OF FOUR PHENOLIC ESTROGENS

No. of experiments	1	2	3	4	5	6	7	8	9	10	11	12
R_1	0.24	0.20	0.22	0.19	0.19	0.19	0.20	0.20	0.25	0.21	0.22	0.18
R_2	1.9	1.8	1.9	1.8	1.5	1.8	1.8	1.8	1.6	1.8		

R_1—The relative potency of estrone to estradiol-17β is expressed as the inverse ratio of the amounts of these compounds required to produce a uterine weight increase of 5 mg.
R_2—The relative potency of estriol to 16-*epi*estriol is expressed as the inverse ratio of the amounts of these compounds required to produce a uterine weight increase of 5 mg.

TABLE 6. URINARY EXCRETION OF PHENOLIC ESTROGENS IN WOMEN WITH NO CANCER

Cases and age	Endocrine status	Estrogen excretion μg./24 hours			
		Estradiol	Estrone	Estriol	16-*Epi*-estriol
L.G. 25	14th day of cycle	5.4	10.0	5.0	5.0
P.D. 25	7th day of cycle	0.7	3.5	6.0	7.2
F.F. 31	6-month pregnancy	10.9	44.8	39.0	80.0
M.G. 57	Postmenopausal (10 years)	0.3	0.88	<0.03*	<0.06*

* Amount of these estrogenic compounds in the urine extracts was not large enough to cause any uterine weight increase.

tions in the urinary excretion of the estrogens are listed in Tables 7 and 8. In both cases there was a rapid reduction in the excretion of all estrogens following surgical castration. The estrone value however returned to a higher level within a period of a few months, whereas estriol and 16-*epi*-estriol remained at a low level or they were no longer detected. The estrone and estradiol-17β values were further reduced in case NW following adrenalectomy.

Recoveries of estrogens added to hydrolyzed urine: To test the extraction, purification and finally bioassay determination a series of recovery experiments were performed in which 1.5 μg. of estriol, 2.0 μg. of 16-*epi*estriol, 1.0 μg. of estrone and 0.5 μg. of estradiol-17β were added separately to four different aliquots (500 ml. each and were hydrolyzed by enzymatic method)

TABLE 7. CHANGES IN URINARY EXCRETION OF PHENOLIC ESTROGENS IN A WOMEN WITH METASTATIC CANCER OF THE BREAST FOLLOWING THE REMVOAL OF ENDOCRINE GLANDS

Endocrine status	Estrogen excretion, μg./24 hours			
	Estrone	Estradiol	Estriol	16-*Epi*estriol
Precastration (Premenopausal)	3.5	0.5	2.9	0.3
7 days postcastration	2.8	0.4	0.3	<0.06*
4 months postcastration	7.6	0.6	<0.03*	<0.06*
1 year postcastration	7.0	0.4	<0.03*	<0.06*
2 months after adrenalectomy	1.4	0.09	<0.03*	<0.06*

Subject NW was a 35-year old woman.
* Amount of these estrogenic compounds in the urine extract was not large enough to cause any uterine weight increase.

TABLE 8. CHANGES IN URINARY EXCRETION OF PHENOLIC ESTROGENS IN A WOMAN WITH CANCER OF THE BREAST FOLLOWING OVARIECTOMY

Endocrine status	Estrogen excretion μg./24 hours			
	Estrone	Estradiol	Estriol	16-*Epi*estriol
Precastration (Premenopausal)	7.7	0.4	5.7	1.3
7 days after castration	0.5	<0.005	<0.03*	0.5
11 months after castration	2.8	0.8	0.7	1.4
13 months after castration	8.9	0.3	0.8	1.2

Subject CC was a 36-year old women.
* Amount of these estrogenic compounds in the urine extracts was not large enough to cause any uterine weight increase.

TABLE 9. COMPARISON OF YIELDS OF ESTROGEN FROM URINE BY ENZYMATIC AND ACID HYDROLYSIS

Method of hydrolysis	Duration of hydrolysis	Amount of estrogen per 24 hours (in terms of estrone)
1. Acid hydrolysis Urine acidified with conc. (12N) HCL (usually 10% of the urine volume) to PH <1 Heat on steam bath 2 or 3 hours	2 hours	18.0 μg.
	3 hours	17.5 μg.
2. Enzymatic hydrolysis β-Glucuronidase 300 U./100 cc. urine Incubate at 37° C.	86 hours	22.0 μg.
	62 hours	20.7 μg.
	48 hours	21.6 μg.
3. Residual urine from the enzyme hydrolysis 2 Acidified to pH 1 with HCL Let stand at room temperature	24 hours	15.0 μg.

Subject is a normal female, age 25, 14 days of the cycle.

from two pooled urines of two postmenopausal women following bilateral adrenalectomy. Blank determinations were made at the same time in each case. The percentage of recovery, calculated from the amount of estrogen recovered after subtracting the urine blank values, are shown in Table 10.

TABLE 10. RECOVERIES OF ESTROGENS FROM ENZYME-HYDROLYZED FEMALE URINE. POOLED URINES OF TWO WOMEN—POSTMENOPAUSAL AND POSTADRENALECTOMY

Amount of estrogens added/500 cc. urine	Amount recovered μg. and % of recovery			
(I) RQ	Estrone	Estradiol-17β	Estriol	16-*Epi*estriol
Blank (RQ_0)	0.07 μg.	0.029 μg.	0.23 μg.	0
RQ_1 1.0 μg. Estrone	0.55 μg. 48%	0.071 μg.		
RQ_2 0.5 μg. Estradiol-17β	0.065 μg.	0.29 μg. 52%		
RQ_3 1.5 μg. Estriol			0.89 μg. 44%	0.08 μg.
RQ_4 2.0 μg. 16-*Epi*estriol			0.5 μg.	0.75 μg. 37%
(II) MB Blank (MB_0)	0.07 μg.	0.02 μg.	0	0
MB_1 1.0 μg. Estrone	0.71 μg. 64%	0.18 μg.		
MB_2 0.5 μg. Estradiol-17β	0.072 μg.	0.36 μg. 68%		
MB_3 1.5 μg. Estriol			0.95 μg. 63%	0.12 μg.
MB_4 2.0 μg. 16-*Epi*estriol			0.10 μg.	1.0 μg. 50%

Estrogens were added to urines after enzymatic hydrolysis.

Recoveries are 48 and 64% for estrone; 52 and 68% for estradiol; 37 and 50% for 16-*epi*estriol and 44 and 63% for estriol.

Recovery of estrogens applied directly on paper: To test the procedures involved in the paper chromatography, recovery experiments were done with the application of pure compounds on the paper and then they underwent the procedures of chromatographic separation and final bioassay determination. The percentage of recoveries as shown in Table 11 are 132 and 140% for estrone, 76 and 80% for estradiol, 97 and 104% for estriol and 34 and 35% for 16-*epi*estriol.

TABLE 11. RECOVERIES OF ESTROGENS APPLIED DIRECTLY ON PAPER

Amount of estrogens applied	Amount recovered-μg. and % of recovery		
(I) Estrone 0.5 μg.	0.70 μg.	or	140%
Estradiol-17β 0.25 μg.	0.20 μg.	or	80%
Estriol 0.75 μg.	0.73 μg.	or	97%
16-*Epi*estriol 1.0 μg.	0.34 μg.	or	34%
(II) Estrone 0.5 μg.	0.66 μg.		132%
Estradiol-17β 0.25 μg.	0.19 μg.		76%
Estriol 0.75 μg.	0.78 μg.		104%
16-*Epi*estriol 1.0 μg.	0.35 μg.		35%

Estrone and estradiol-17β were applied together in one spot on paper and underwent chromatography in n-heptane: CH_3OH system; elution and then bioassay. Estriol and 16-*epi*estriol were applied together in one spot on paper and underwent chromatography in Bush B_8 system; elution and then bioassay.

DISCUSSION

By the use of paper chromatography, a complete separation of four phenolic estrogens in a sufficient state of purity has been accomplished. This was shown by the exact correspondence between the positions of pure estrogens on the parallel runs and their respective positions of estrogens from the urine extract on the paper, ascertained by the result of bioassay. The results indicated that the separation of estrogens in urine extract was as complete as the separation of pure compounds (Figs. 4 and 5).

There were numerous difficulties in obtaining good chromatograms of urine extracts. Efforts have been made continuously to improve the technique. The urine contains a great amount of lipid impurities which are the main causes of spreading of spots and streaking. To remove these impurities has proved difficult without simultaneously removing the estrogens. Acid hydrolysis not only causes the formation of emulsions, but we have found that it also destroys a considerable amount of estrogen (10 to 20%). Hydrolysis with β-glucuronidase is definitely superior for it gives a higher yield and much cleaner residue which greatly decreases the tendency to spread on the chromatogram (Table 9).

The amount of urinary extract applied on the paper must not be too large on one spot. Large spots will cause streaking and this will interfere with the separation of the estrogens. It must be remembered, however, that the amount of extract applied on the spot must contain enough hor-

mone to produce a significant increase of mouse uterine weight (calculated $P<0.01$) in the bioassay. Application of the urine extract on multiple spots is the technique of choice.

Bioassay, though time consuming and expensive, proves to be still the best method in the quantitative determination of microquantities of estrogens.

Measurement of Kober's chromogen is not only non-specific for estrogens but it is not sensitive enough to measure a very small amount of estrogens in the urine of castrated and postmenopausal women. The limits of accu-

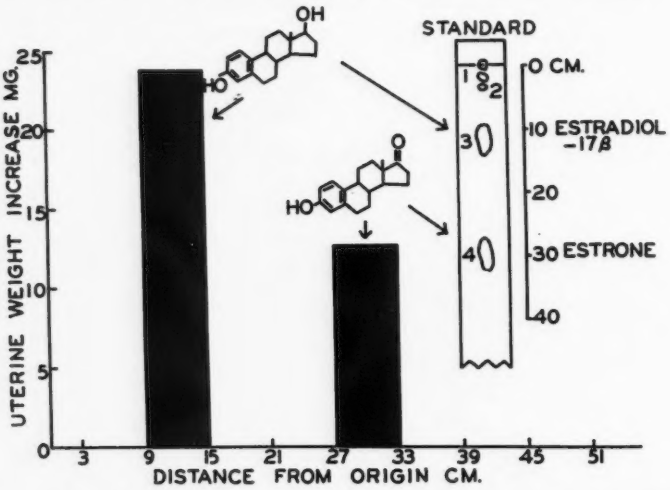

FIG. 4. Demonstration of the complete accordance between the positions on the standard chromatorgram of estradiol-17β and estrone and the presence of estrogenic activity of eluates from the center strip containing these estrogenic compounds from the urine extract. Note: The bioassay of intermediate strips showed no estrogenic activity. Spots 1 and 2 are estriol and 16-*epi*estriol respectively.

racy achieved by Kober reaction are 5 μg. of estrogen per 24 hours urine specimen (3, 4). Moreover, the urine contains large amounts of non-estrogenic colored materials. Even with extensive purification procedures, a considerable amount of colored material will still remain. This interferes with the final color development. Greater sensitivity in measuring the estrogens may be obtained by the use of fluorometric method but they are susceptible to instability and quenching by solvent or urinary extracts (12, 13).

The recoveries of estrogens added to the hydrolyzed urine were indeed low in two experiments. With the exception of 16-*epi*estriol the considerable loss of estrogens in these experiments appears to have occurred in the extraction procedures since recoveries were between 76 and 140% with pure estrogens applied directly on the paper and subsequently underwent

chromatography, elution and bioassay. The especially low recoveries of 16-*epi*estriol in both recovery experiments is now under further investigation. Although it is speculative at present to formulate any theory to explain the low recoveries of 16-*epi*estriol, it can be attributed to either of the two possibilities: (1) the presence of small quantities of very active estrogens in 16-*epi*estriol. These contaminants would be removed by the chromatographic process and hence would result in the lower bioassay after chromatography, and (2) the 16-*epi*estriol was oxidized and rendered inactive during chromatography.

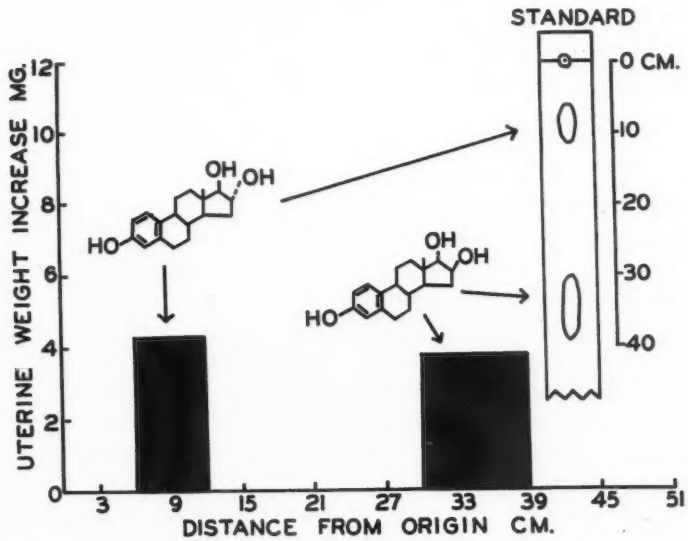

FIG. 5. Demonstration of complete accordance between the positions on the standard chromatogram of estriol and 16-*epi*estriol and the presence of estrogenic activity of eluates from the center strip containing these estrogenic compounds from the urine extract. Note: Bioassay of the intermediate strips showed no estrogenic activity.

Whether the loss of estrogen in these recovery experiments implies that there is a similar loss in the process for the determination of these compounds in the urine extracts cannot be ascertained. Results from Brown's paper (3) showed that in the early proliferative phase of the menstrual cycle of a normal female, the excretion of estrone was 5.1 μg.; of estradiol-17β, 0; and of estriol, 6.0 μg./24 hours. Bauld's paper (4) showed that in a 35-year old normal female, ninth day of cycle, the excretion of estrone was 7.0 μg.; of estradiol-17β, 3.5 μg.; and of estriol, 27.0 μg./24 hours urine. It seems that the magnitude of these values does not differ greatly from that of our results. However, these values are hardly comparable because of the difference of methods used. Furthermore, our results presented in this paper

represent only estrogens from the hydrolysis of the estrogen glucuronides; the amount of estrogens from estrogen sulfate which was obtained after acid hydrolysis was not included. The values of urinary estrogens obtained from the combination of both enzyme and acid-hydrolyzed urine will be considerably higher.

It is interesting to note that in both of our recovery experiments there is an increase of estradiol-17β recovery in samples in which estrone was added. Likewise, there was an increase in the recovery of 16-*epi*estriol where estriol was added and similarly an increase in estriol when 16-*epi*estriol was added to the urine. The cause of this phenomenon is not clear. Our samples of estriol were known to be impure since they contained traces of 16-*epi*estriol, but the samples of estrone, estradiol-17β and 16-*epi*estriol were homogenous as determined by paper chromatography.

SUMMARY

A method for clean separation of four phenolic estrogens in human urine has been presented. This method involves enzymatic hydrolysis, ether extraction, paper chromatographic separation, purification and finally bioassay.

The specificity and reliability of the bioassay of estrogens have been critically appraised. Biological assays of estrone and estradiol-17β are satisfactory. Assay of two impeded estrogens, estriol and 16-*epi*estriol are less reliable, but multiple level assay can increase the accuracy.

In recovery experiments in which estrogens were added to urine, not all of these added compounds were recovered, the deficit amounting to 30 to 50% of the amount added. It seems that the loss occurred during the extraction procedure. Washing with $NaHCO_3$ may remove amounts of estriol and 16-*epi*estriol.

All results of urinary excretion of estrogens described in this paper were obtained from the urine following enzyme hydrolysis. The portion following further acid hydrolysis has not been discussed here. The actual values will therefore be higher.

Acknowledgment

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PARATHYROID FUNCTION AS STUDIED BY CONTINUOUS PERITONEAL LAVAGE IN NEPHRECTOMIZED RATS[1]

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RECENT work utilizing nephrectomized animals (1, 2) has added convincing proof to an old argument that the parathyroids are able to exert their influence on calcium metabolism even in the absence of the kidneys. Current interest in the relationship of citric acid and calcium metabolism (3–6) has given rise to an increasingly popular concept that parathyroid influence is exerted through citric acid metabolism of bone. Concurrently with these advances in the study of the action of this hormone, interest has been aroused in the ability of bone to supply calcium to the extracellular fluid in maintenance of constant plasma levels of this ion. Pioneering in this concept were McLean and Urist (7), who emphasized that bone was able to supply calcium to the extracellular fluid compartments in the absence of the parathyroids. This has been developed further by work from this laboratory in which the relationships of calcium and phosphate ions with and without the influence of the parathyroids were discussed (8). Recently, Copp (9), by continuous infusion of ethylenediamine-tetra-acetic acid (versene) to normal and parathyroidectomized dogs, compared the ability of bone to supply calcium to extracellular fluid spaces under these two conditions.

In the work reported below, bone and extracellular fluid relationships were studied by the production of "low calcium stress" in nephrectomized rats. By the use of continuous peritoneal lavage, utilizing a calcium-free isotonic wash solution, the ability of bone to supply calcium continuously both in the nephrectomized and nephrectomized-parathyroidectomized rat could be studied and, by histological examination, the effect of this "stress" on bone could be examined. It is hoped that the results given below will throw some light on the physical and physiological phenomena concerned in the maintenance of plasma calcium levels.

MATERIALS AND METHODS

Male Holtzman rats, weighing between 200 and 250 gm. were used in these experiments. Peritoneal lavage was accomplished by an indwelling inguinal catheter using a slightly modified procedure of the technique described by Talmage *et al.* (10). The lavage rinse consisted of .84% NaCl plus 1.5% glucose maintained at 40° C in a water bath. (In the discussions to follow the word "rinse" is used to refer to the fluid placed in

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the animal, the word "wash" to refer to the fluid removed from the peritoneal cavity.) The animals were kept under light nembutal anesthesia throughout the experimental period. At the conclusion of the experiment, the femurs, mandibular condyles, and parathyroid glands were removed for histological preparation. Bone was decalcified by repeated changes of acid Bouins at 37° C over a two-week period. Calcium analyses were made by flame photometry, and citric acid determinations by the Hunter and Leloir modification (11) of the method of Pucher *et al.* (12).

Experimental Procedure: For each experiment the following basic pattern was used: Animals previously maintained on a calcium-deficient diet for two days were bilaterally nephrectomized. Three to six hours later the parathyroids were removed individually (only in the first series of experiments were the parathyroids removed the following day, as described below). The following morning, catheters were inserted through the inguinal canals and the peritoneal lavages commenced. For these washes, 30 ml. of the rinse was placed in each animal and immediately removed. A second 30 ml. was placed in the cavity and allowed to equilibrate for a given length of time. The wash was then removed for analysis, the animal was again rinsed with 30 ml. of fluid which was discarded, and an additional 30 ml. was introduced into the animal for the succeeding equilibration period. This procedure was continued throughout the experimental period, the times of equilibration varying for the purpose of the experiment. In every experiment both nephrectomized only and nephrectomized-parathyroidectomized rats were used.

RESULTS

A. *Wash levels during 8 hrs. of continuous peritoneal lavage*

In a previous report (2) the consistency of the citric acid and calcium levels of peritoneal washes taken over a three day period was noted, as was the immediate drop in these values produced by parathyroidectomy in nephrectomized rats. In that study a peritoneal plug was used and washes were made as needed up to three times daily. The wash after one hour of equilibration averaged between 6.3 and 6.8 mg. per 100 ml. for animals with intact parathyroids and between 3.5 and 3.9 mg. per 100 ml. for their parathyroidectomized mates. For comparative purposes, in the present study, a group of experiments were run in which a continuous peritoneal lavage was maintained for eight hours with one hour equilibration periods. In contrast to the previous study, the peritoneal cavities were rinsed between each equilibration period and the animals were maintained under light nembutal anesthesia throughout. To show again the speed with which the equilibration level changed after parathyroidectomy, these glands were removed during the first half hour of the second equilibration period. The calcium and citric acid levels for the hourly periods are given in Table 1. The wash level of the control rats remained at approximately 5.0 mg. per 100 ml. throughout the experimental period, while the citric acid concentration dropped steadily. The effect of parathyroidectomy on both the citric acid and calcium levels can be seen by one half hour after removal of the glands. After the initial drop, the calcium levels remained fairly constant throughout the experiment. In contrast to the maintenance of consistent calcium levels throughout this period, the citric acid levels

TABLE 1. HOURLY CALCIUM AND CITRATE LEVELS OF THE RINSE REMOVED FROM PARATHYROIDECTOMIZED & CONTROL RATS UNDERGOING CONTINUOUS PERITONEAL LAVAGE

	Condition	Hourly equilibrium periods							
		1	2	3	4	5	6	7	8
Control	Ca	5.0 ± .16	4.9 ± .23	4.7 ± .13	4.8 ± .24	5.0 ± .13	5.2 ± .16	5.1 ± .20	5.4 ± .03
	Cit	.82± .04	.70± .03	.64± .05	.56± .05	.47± .04	.42± .03	.40± .03	.34± .03
PTX[2]	Ca	4.9 ± .11	3.8 ± .3	2.9 ± .1	3.0 ± .1	3.1 ± .02	3.3 ± .1	3.4 ± .14	3.6–
	Cit	.86± .03	.53± .03	.47± .02	.42± .01	.36± .02	.31± .02	.33± .02	.36–

Notes: 1) All values given in mg./100 ml. with standard error.
2) PTX—animals parathyroidectomized during the first half hour of Period 2.
3) Each group consisted of five animals.

drop further and level off at approximately one half of their starting values, with the differences between the parathyroidectomized and control animals gradually diminishing. As yet, no explanation can be given to the significance of this relative loss of citric acid in the presence of steady calcium levels.

B. *Rate of calcium addition to the extracellular fluid compartments*

In order to compare the ability of parathyroidectomized and nonparathyroidectomized rats to furnish calcium to the extracellular fluid under these "stressed" conditions, equilibration periods of from 10 minutes to 2 hrs. were used. The experiments were arranged so that both shorter and longer periods of equilibration were made at all stages during the experiments. The data from periods of equal length were averaged and are presented in graph form (Fig. 1). It can be noted that significant differences in the calcium content of the washes were apparent even when left in the animals only ten minutes. While this would seem to indicate that the parathyroids influence the rate at which calcium can be removed from bone as well as the final level of equilibration, the calculations given in Table 2 indicate that the differences in rate are small if any. These calculations are based on the assumption that the ten minute wash periods are the most indicative of the rate at which the bone is able to supply calcium, since at the longer periods existing levels interfere with the addition of further calcium. Using the 10 min. periods, it is calculated that the parathyroidectomized rats supplied approximately 13 mg. of calcium per kilo per hour while their controls supplied 16 mg. per kilo per hour. These data suggest that while the parathyroids may affect the rate of calcium transfer from bone, they are primarily concerned with levels of equilibration.

C. *The effect of "low calcium stress" by peritoneal lavage on bone*

Numerous reports have demonstrated the effect of parathyroid extract on bone histology. Of these, one of the most thorough is that of Heller, *et al.* (13). The work presented here is one of the first to show the effect of experimentally increased endogenous parathyroid activity on bone. Since

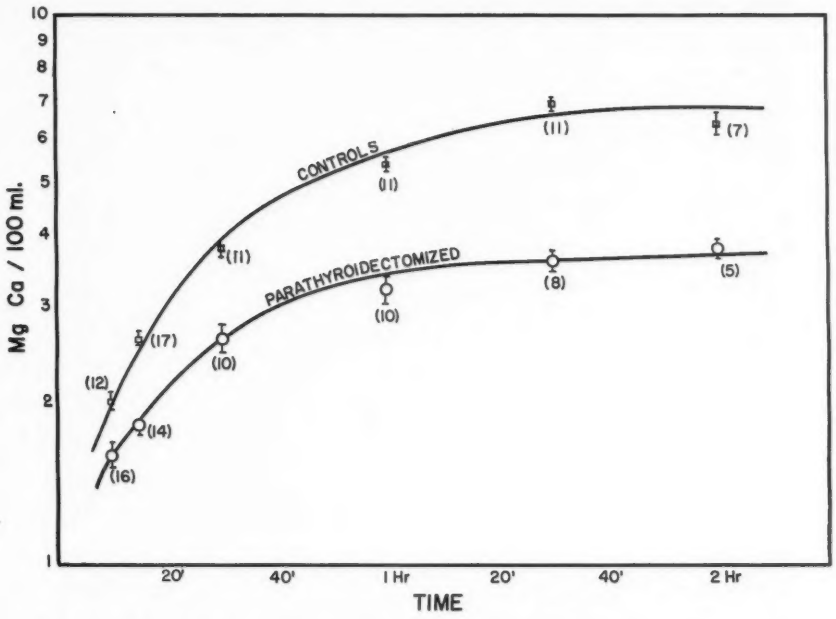


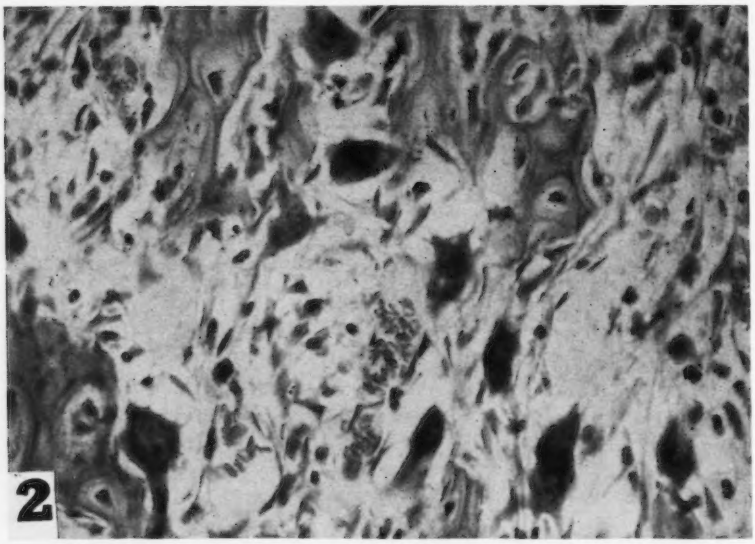
FIG. 1. *Calcium Concentrations of Wash vs. Time of Equilibration:* All points on the graph are given with the S.E. ($\sqrt{\Sigma d^2/n(n-1)}$) represented by vertical bars. Figures in parentheses represent the number of animals used.

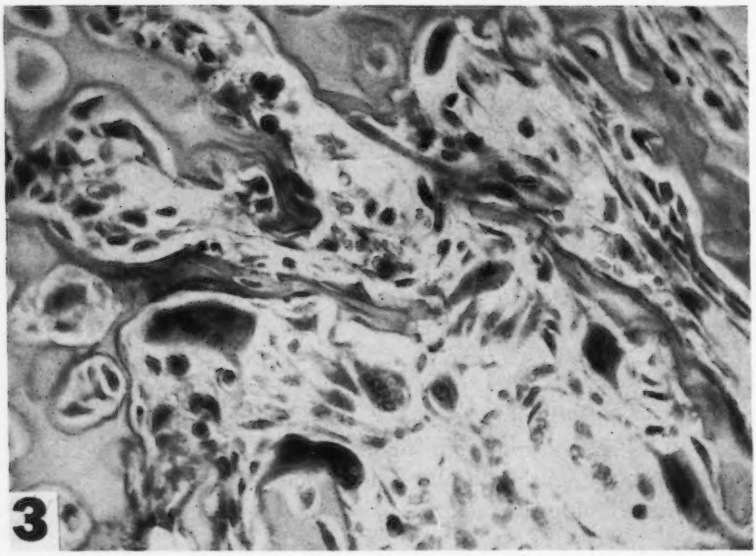
these appear to follow the changes described by Heller *et al.*, no detailed histological description will be given. The purpose of this work is to contrast the bone picture of the parathyroidectomized animal with that of the animal with intact glands. The two bones used were the femur and the condyle of the mandible. In the former, osteoclasts are looked for particularly in the sub-epiphyseal region. At the junction of the periosteum and the sub-epiphyseal plate, these cells are relatively numerous, even in the parathyroidectomized animals. The condyle has been described by Meyers and Reeve (14) as containing far higher ratios of osteoclasts to other bone elements than are found normally in long bones.

In the parathyroidectomized rat there was no detectable increase in the

TABLE 2. CALCULATIONS FOR DETERMINATION OF QUANTITIES OF CALCIUM REMOVED BY PERITONEAL LAVAGE

	Controls	Parathyroidectomized
1. Average calcium value—10′ period	.020 mg./ml.	.016 mg./ml.
2. Fluid removed each wash	30 ml.	30 ml.
3. Total calcium removed each wash	.60 mg.	.48 mg.
4. Calcium removed/hour at 10′ rate	3.60 mg.	2.88 mg.
5. Average weight of animal	225 gm.	225 gm.
6. Calcium removed/hour/kilo at 10′ rate	15.9 mg.	12.9 mg.





EXPLANATION OF PLATE

FIG. 2. Femur from nephrectomized rat maintained on a calcium free wash showing portions of 12 osteoclasts. H and E ×400.

FIG. 3. Mandibular condyle from nephrectomized rat maintained on a calcium free wash. Note numerous portions of osteoclasts. Large osteoclasts in the subcartilage area (left of picture) represent normal condition; deeper osteoclasts (right of picture) found only in stressed animals. H and E ×400.

numbers of osteoclasts in either bone, even after 12 hours continuous peritoneal lavage. This phenomenon is of particular interest when it is correlated with the continual low level addition of calcium to the extra-cellular fluid that occurs in these animals throughout the stressed period. In the animal with intact parathyroids, the increase in numbers of osteoclasts over an 8 to 12 hour period of "calcium stress" is most striking (Fig. 2, 3). In the femur this is most easily seen in the subepiphyseal region or zone of reorganization of the diaphysis (Fig. 2). Osteoclasts of all sizes are abundant and are frequently found in clusters around portions of bone trabeculae. In the mandibular condyle a concentration of osteoclasts is always found at the base of the cartilaginous plate. This area is affected neither by parathyroidectomy nor by stress. However, in the "stressed" animal with intact parathyroids, increased numbers of osteoclasts can be found throughout the bone, deep in the neck of the condyle, in areas consisting mostly of compact bone.

DISCUSSION

The use of continuous peritoneal lavage with a calcium-free isotonic rinse has permitted the study of a condition in rats which we have termed "low calcium stress." By this technique, the bones of animals which have been maintained on a calcium deficient regime have been called upon to furnish calcium at near maximum rates for periods up to 12 hrs. Since all animals were nephrectomized 24 hrs. before use, this procedure has provided an excellent opportunity to study the ability of the parathyroids to stimulate the release of calcium from bone to the extracellular fluid in a situation in which any influence of the kidney or of intestinal absorption of calcium has been ruled out.

It seems quite obvious that in the absence of any activity of the parathyroids, the bone is capable of continuously supplying calcium over long periods of time to the fluid compartments. The equilibrium level between bone and extracellular fluid in the parathyroidectomized condition is between 3 and 4 mg. calcium per 100 ml., most of which is assumed to be in the ionized form. These experiments indicate that fluctuations in numbers of osteoclasts play only a minor role, if any, in this equilibrium process. Despite the continuous lavage for up to 12 hours, no indication has yet been noted of any increase (or decrease) in the number of osteoclasts in the two type of bones studied. One might suggest that this equilibrium may be an entirely physical phenomenon. Such could be the case, if at the normal pH of body fluids the apatite crystals of bone were soluble to this extent.

It is equally apparent that the hormone of the parathyroids is capable of raising the equilibrium level for calcium between bone and the extracellular fluid compartments, and that under these "stress" conditions this is done in the presence of increased numbers of osteoclasts. In these experiments there was a marked increase in numbers of osteoclasts in the regions

of the bone studied in animals with functioning parathyroids. This cannot, however, be construed as evidence that the parathyroids function solely by increasing the numbers of osteoclasts. Fluctuations in calcium levels due to changes in hormone titers can occur in minutes (2), and in these experiments, proper calcium concentrations were maintained even during the initial period of the continuous lavage stress, whether that period was of 15 minutes or of an hour's duration. It would appear to follow, therefore, that in the normal condition, the animal is immediately able to cope with fluctuations in calcium supplies without the necessity of first altering the numbers of bone cell components.

This laboratory, along with others, has shown the close connection between citric acid and the influence of the parathyroids on calcium metabolism. Following the lead of Neuman *et al.* (5), we would suggest further that the increased production of citric acid discussed by these authors is somehow related to the function of osteoclasts. Realizing that the evidence is still incomplete, one might, however, suggest that one function of the hormone of the parathyroid is to release citric acid immediately from bone cells, possibly the osteoclasts, for the localized dissolution of bone, and thus to raise the calcium of the fluid compartments to the required levels. In cases of increased endogenous production, such as "stress" conditions, or after exogenous administration, the hormone is capable, either directly or indirectly, of increasing the numbers of osteoclasts as a result of, or to meet, the increased demand for calcium.

SUMMARY

The influence of the parathyroids on the ability of bone of nephrectomized rats to supply calcium to the extracellular spaces has been studied by means of continuous peritoneal lavage. Isotonic, but calcium and phosphate-free, rinses were allowed to equilibrate in the peritoneal cavity of each rat for varying periods of time, and then removed for analysis. Parathyroidectomy after the peritoneal lavage had commenced produced a drop in both calcium and citric acid levels of the wash within half an hour. However, the parathyroidectomized animals were able to maintain a continuous and constant calcium output from bone after the initial drop. While differences between control and parathyroidectomized animals could be seen even in the 10 minute equilibration periods, calculations of rate of removal of calcium indicated that the most likely effect of the hormone of the parathyroids was concerned with the level of equilibration of calcium. During the maintenance of the higher calcium levels in the extracellular compartment, bones of animals with intact parathyroids showed a marked increase in the number of osteoclasts after as little as six hours of continuous peritoneal lavage. Parathyroidectomized rats showed no such increase. Problems related to this dual mechanism of calcium release from bone are discussed.

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THE EFFECTS OF SEX HORMONES ON THE SUCCINOXIDASE ACTIVITY OF THE ADRENAL GLAND OF THE RAT[1]

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IT HAS been reported previously (1) that the succinoxidase activity of the adrenal glands of adult male rats is approximately 80% higher than that of female animals of the same age. We have suggested that this sex difference is dependent on gonadal secretions because it was considerably smaller in sexually immature animals. The experiments reported here were performed to elucidate further the nature of this sex difference. They are concerned with the effects of gonadectomy and of administration of sex hormones on the succinoxidase activity of the adrenal glands of adult rats of both sexes. The oxygen uptake of adrenal gland slices of male and female rats has also been measured, in order to see whether the difference observed in homogenates reflected a difference in the metabolic behaviour of the intact organized tissue.

METHODS

Succinoxidase activity of the adrenal gland. The experiments were performed in male and female rats of the Wistar strain bred in the Department of Physiology. Gonadectomy was performed under ether anesthesia at 4–5 weeks of age. The control animals were sham operated at the same time. The animals were kept in group cages for a further 6 weeks so that their ages at the time of killing ranged between 10 and 11 weeks. Sex hormones were administered by daily subcutaneous injections in 0.1 ml. of sesame oil for two weeks just prior to killing. The control animals received daily subcutaneous injections of 0.1 ml. of sesame oil for the same length of time. Testosterone propionate,[2] estradiol dipropionate[2] and progesterone[2] were administered in daily doses of 2.5, 0.25 and 0.5 mg. respectively. All animals were fed fox breeder cubes (Master Feeds) and water *ad libitum*.

The killing of the animals, removal of the adrenal glands, and method of determination of the succinoxidase activity and nitrogen content were the same as previously described (1) except that the glands were homogenized for 1.5 minutes instead of 3 because more consistent results were obtained in this fashion. The incubation mixture for the determination of the succinoxidase activity contained, in addition to the reagents listed in the previous communication, 0.3 ml. of $4\times10^{-3}M$ $CaCl_2$. Through an error this was not stated in the previous report although it had been used. In all experiments the adrenal glands of two animals were used for each determination.

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[2] Liberal amounts of testosterone propionate (Perandren), estradiol dipropionate (Di-ovocylin) and progesterone (Lutocylin) were kindly supplied by Dr. C. W. Murphy of the Ciba Company of Canada, Montreal.

The results have been expressed in terms of μl. of oxygen used during the first hour of incubation per 100 mg. of fresh gland weight, per 1 mg. of nitrogen and per pair of glands. The first two expressions are referred to subsequently as relative enzyme activity, while the third is referred to as total activity.

Oxygen uptake of adrenal gland slices. The animals were selected from the colony according to their body weights so that their ages would be approximately the same. All animals received food and water *ad libitum*. The animals were killed and the adrenal glands removed and weighed as described previously (1). The glands were placed on a buffer-soaked piece of filter paper laid on a covered petri dish filled with ice. Each gland was cut with a razor blade into four parallel slices. The slices thus obtained from each animal were placed in Warburg vessels containing 2.0 ml. of Krebs-Ringer phosphate buffer (2). The substrates used were 30*M* sodium succinate or 90*M* glucose. The pH of the incubation medium was adjusted to 7.4. The center well contained 0.2 ml. of 10% KOH and a piece of filter paper 2.5 cm. square. The vessels were equilibrated for 15 minutes at 38° C. The gas phase was 100% oxygen. The results have been expressed as μl. of oygen per 100 mg. of fresh adrenal gland weight.

RESULTS

Effect of gonadectomy. The results are summarized in Table 1. The results obtained in the control male and female animals were essentially the same

TABLE 1. THE EFFECT OF GONADECTOMY ON THE SUCCINOXIDASE ACTIVITY OF THE ADRENAL GLANDS OF MALE AND FEMALE RATS*

Animals	No. of animals (No. of observations)	Body wt., gm.	Adrenal gland			μl. O_2 in 1st hour		
			Wt. mg.	Wt. Mg./ 100 gm. Body wt.	% N	Per 100 mg. fresh wt.	Per mg. N	Per pair of glands
Normal Male	22 (11)	227±9	26.5±0.7	11.8±0.3	2.40±0.05	808±31	336±11	215±10
Castrated Male	20 (10)	200±8	30.4±1.0	15.6±0.7	2.40±0.07	537±21	225±10	163± 7
Normal Female	20 (10)	166±4	37.8±1.4	22.8±0.7	2.39±0.02	385± 9	162±12	146± 9
Ovariectomized Female	24 (12)	179±5	32.1±1.2	18.2±0.5	2.35±0.10	504±19	224±18	163± 7

* All results in this and in the following tables are given as Mean ± S.E.

as those reported previously (1), *i.e.* the succinoxidase activity of the adrenals (both relative and total) in the young adult males was considerably higher than that of the females, in spite of the fact that the adrenals of the females were the heavier. The percentage of nitrogen was the same in the adrenals of all groups. The sex differences in succinoxidase activity and in size of the adrenals were completely suppressed by gonadectomy. This was due to a significant decrease ($P < 0.01$) in the succinoxidase activity of the glands of the castrated males and to a significant increase in the enzyme activity of the adrenals of the ovariectomized females ($0.02 > p > 0.01$). The same is true with respect to the effect of gonadectomy on adrenal weight, *i.e.* the normal difference disappeared as a result of a significant increase in the weights of the glands in the castrated males ($p < 0.01$) and of a significant decrease ($P < 0.01$) in adrenal weight in the ovariectomized females.

The effect of ovariectomy on the absolute weight of the adrenal glands has not proven to be consistent. Thus, in a subsequent experiment no significant difference was observed, while in a third experiment a significant

TABLE 2. THE EFFECT OF OVARIECTOMY ON THE SUCCINOXIDASE ACTIVITY OF THE ADRENAL GLANDS

Animals	No. of animals (No. of observations)	Body wt., gm.	Adrenal gland			μl. O_2 in 1st hour		
			Wt. mg.	Wt. mg./ 100 gm. Body wt.	% N	Per 100 mg. fresh wt.	Per mg. N	Per pair of glands
Normal Female	58 (29)	158±2	36.9±0.9	23.5±0.5	2.47±0.02	365±13	148± 6	134±5
Ovariectomized Female	58 (29)	187±3	36.9±1.0	19.7±0.3	2.50±0.06	467±14	192±10	172±5

increase in the absolute weight of the glands was found. In both these cases however, there was a significant decrease in the relative adrenal gland weights (expressed in terms of mg. per 100 gm. of body weight).

Because of the variability in response to ovariectomy in these three experiments the results were pooled and have been summarized in Table 2. The following conclusions seem justified: (1) the final body weights of the ovariectomized females were significantly higher ($P<0.01$) than those of the normal females; (2) there was no difference in the absolute adrenal weights, but the gland weights expressed per 100 gm. of body weight were significantly lower in the ovariectomized than in the normal females ($P<0.01$); (3) the succinoxidase activity of the adrenals was approximately 30% higher in the ovariectomized than in the normal females ($P<0.01$).

Effect of administration of testosterone propionate to castrated males. The results are presented in Table 3. A slight but significant decrease in the weight of the adrenal glands ($0.05>P>0.02$), and a slight but also significant increase in the percentage of nitrogen ($P<0.01$) of the glands can be noted. The most striking effect, however, was exerted upon the succinoxidase activity of the adrenals. The activity in the androgen-treated animals was more than double that in the controls ($P<0.01$). Thus, the administration of testosterone propionate to castrated males reversed completely the effects of castration.

Effect of administration of estradiol dipropionate to ovariectomized females. The results are summarized in Table 4. The final body weight of the estrogen-treated animals was significantly lower than that of the controls ($P<0.01$). Both the absolute and relative adrenal gland weights were significantly higher in the estrogen treated than in the control animals ($P<0.01$ in both instances). The percentage of adrenal nitrogen was slightly (8%) but significantly higher in the estrogen-treated group ($P<0.01$).

TABLE 3. THE EFFECT OF TESTOSTERONE PROPIONATE ON THE SUCCINOXIDASE ACTIVITY OF THE ADRENAL GLANDS OF CASTRATED MALE RATS

Animals	No. of animals (No. of observations)	Body wt., gm.	Adrenal gland			μl. O_2 in 1st hour		
			Wt. mg.	Wt. mg./ 100 gm. body wt.	% N	Per 100 mg. fresh wt.	Per mg. N	Per pair of glands
Castrated Male	18 (9)	200±6	31.8±1.2	15.9±0.6	2.32±0.04	403±15	174± 7	129±7
Castrated Male+Testosterone propionate	20 (10)	198±5	28.8±0.6	14.6±0.3	2.46±0.02	990±23	404±12	286±9

TABLE 4. THE EFFECT OF ESTRADIOL DIPROPIONATE ON THE SUCCINOXIDASE ACTIVITY OF THE ADRENAL GLANDS OF OVARIECTOMIZED RATS

Animals	No. of animals (No. of observations)	Body wt., gm.	Adrenal gland			μl. O_2 in 1st hour		
			Wt. mg.	Wt. mg./100 gm. body wt.	% N	Per 100 mg. fresh wt.	Per mg. N	Per pair of glands
Ovariectomized Female	24 (12)	187±6	36.5±1.4	19.5±0.5	2.37±0.02	386±13	163±5	140±6
Ovariectomized Female +estradiol Dipropionate	24 (12)	145±5	45.0±1.4	31.5±1.2	2.56±0.02	447±15	175±6	202±9

The effect of estradiol dipropionate on the adrenal succinoxidase activity was small. The activity was 15.8% higher in the test group when the results were expressed per unit of fresh weight ($P < 0.01$), but it was not significantly different in the case of the results expressed per unit of adrenal nitrogen. However, the succinoxidase activity per pair of glands was appreciably increased (44%) by the estrogen-treatment as a result of increases both in activity per unit of fresh weight and in total weight of the adrenal glands.

Effect of administration of progesterone to ovariectomized females. The results are presented in Table 5. Progesterone, at the dose used, had no effect whatsoever on body weight, adrenal gland weight, adrenal nitrogen content or succinoxidase activity.

The oxygen comsumption of adrenal gland slices of adult male and female rats in the presence of succinate or of glucose as substrates. The adrenal gland slices of the male rats showed a higher oxygen consumption than those of the females in the presence of both succinate and glucose (Table 6). It will be noted (Fig. 1) that the difference becomes more marked with time because the rate of oxygen uptake, particularly in the presence of succinate, decreased faster in the female than in the male adrenal slices. The oxygen uptake at the end of two hours was 41% higher in the males

TABLE 5. THE EFFECT OF PROGESTERONE ON THE SUCCINOXIDASE ACTIVITY OF THE ADRENAL GLANDS OF OVARIECTOMIZED RATS

Animals	No. of animals (No. of observations)	Body wt., gm.	Adrenal gland			μl. O_2 in 1st hour		
			Wt. mg.	Wt. mg./100 gm. body wt.	% N	Per 100 mg. fresh wt.	Per mg. N	Per pair of glands
Ovariectomized Female	24 (12)	182±4	34.7±1.2	19.1±0.5	2.59±0.03	412±15	159±7	146±6
Ovariectomized Female +Progesterone	24 (12)	184±5	33.1±1.2	18.0±0.5	2.59±0.04	409±14	158±6	136±6

TABLE 6. THE OXYGEN CONSUMPTION OF ADRENAL GLAND SLICES OF ADULT MALE AND FEMALE RATS IN THE PRESENCE OF SUCCINATE OR GLUCOSE AS SUBSTRATES

Animals	No. of animals	Substrate	Body wt., gm.	Adrenal wt., mg.	mg./100 gm. body wt.	μl. O_2/100 mg. fresh adrenal wt./2 hrs.
Male	9	Succinate	258±17	32.2±1.5	12.8±0.7	702±21
Female	9	Succinate	190± 9	47.2±2.1	25.2±1.1	496±18
Male	8	Glucose	186± 4	25.9±1.0	13.9±0.4	360±16
Female	8	Glucose	149± 4	37.1±2.5	24.9±1.4	292±12

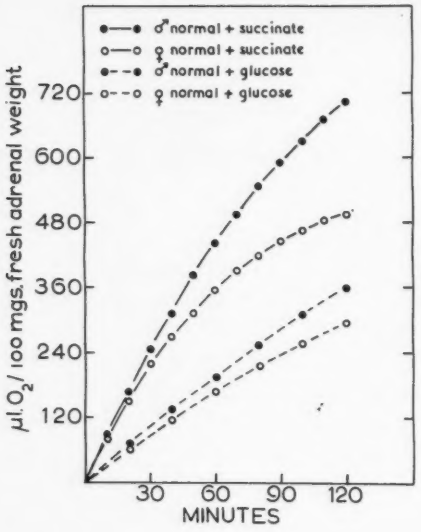

FIG. 1. The oxygen consumption by adrenal gland slices of male and female rats in the presence of glucose or succinate as substrates.

than in the females ($P < 0.01$) when succinate was the substrate; when glucose was the substrate the value was 23% higher in the males ($P < 0.01$).

DISCUSSION

The effects of gonadectomy and of administration of sex hormones on adrenal gland weight have been reviewed many times (3, 4, 5, 6, 7, 8, 9). In general, the results reported here agree with those obtained by other authors.

The sex difference in the succinoxidase activity of whole adrenal gland homogenates reported previously (1) has been confirmed. This difference applies also to the respiration of adrenal gland slices, since the oxygen consumption of slices from male rats incubated in media containing succinate or glucose has been found to be higher than that of similar preparations obtained from female animals. These findings indicate a sex difference in metabolic function of the adrenal gland which is opposite in direction to the sex difference in gland size. It should be noted, however, that under the experimental conditions used, the total oxygen uptake per pair of sliced glands was not different in the two sexes.

The sex difference in both the size and the succinoxidase activity of the adrenal gland is determined by the secretions of the gonads. This is borne out by the fact that these differences disappeared in gonadectomized animals of both sexes. Furthermore, as shown in an earlier article (1), sexually immature animals showed no difference in adrenal gland weight and only a small difference in enzyme activity.

The relative roles played by the secretions of the testes and of the ovaries in determining this sex difference have been partially elucidated. Removal

of the testes resulted in an increase in adrenal weight and in a decrease in succinoxidase activity, whereas the removal of the ovaries gave rise to the opposite changes. The changes in absolute weight of the adrenals after ovariectomy were inconsistent. However, in all cases there was an increase in body weight following ovariectomy, and adrenal growth in these animals did not keep pace with body growth. Therefore the relative weights of the adrenal glands of the ovariectomized females were lower than in the normal females. This is essentially in agreement with the findings reported recently by Carter (9).

The effects of gonadectomy suggest that the secretion of the testis causes a decrease in weight of the adrenal gland and an increase in its succinoxidase activity, whereas the secretion of the ovaries produces an increase in adrenal weight and a decrease in the activity of this enzyme system.

The results obtained after the administration of estradiol dipropionate and of progesterone do not explain the observed effect of ovariectomy on the succinoxidase activity of the adrenals. Thus, when the former substance was administered to ovariectomized female rats a small increase in the relative and a moderate increase in the total activity of this enzyme system was obtained, rather than a decrease. This was accompanied by adrenal enlargement and by a small but significant increase in the nitrogen content of the glands. The results obtained after the administration of progesterone also fail to explain the effects of spaying. Under the conditions used this hormone exerted no effect on adrenal size, nitrogen or succinoxidase activity. It is quite possible that the failure to restore the succinoxidase activity of the adrenal glands of the ovariectomized animals to normal was due to inappropriate dosage since only one dose was tried of estradiol dipropionate and of progesterone. On the other hand it may be that a balanced combination of estrogenic and progestational hormones is required to permit normal oxidative metabolism in the adrenals after removal of the ovaries.

The administration of testosterone propionate to castrated males completely reversed the effects of castration *i.e.* there was a decrease in adrenal gland weight and a large increase in succinoxidase activity.

The significance of the effects of the sex hormones on the succinoxidase activity of the adrenals, in terms of adreno-cortical function, can best be assessed by comparing them with those of substances known to stimulate and inhibit adrenocortical secretion *e.g.* ACTH and cortisone. The experiments reported in the following paper were performed in an attempt to establish the possible significance of the effects of testosterone propionate.

SUMMARY

The effects of gonadectomy and of administration of sex hormones on adrenal gland weight, nitrogen content and succinoxidase activity have been studied in young adult rats. Castration resulted in an increase in

absolute and relative adrenal gland weight and in a reduction in the succinoxidase activity of these glands. Ovariectomy caused no change in absolute adrenal gland weight; since it increased body weight, a decrease in relative adrenal weight occurred. The succinoxidase activity of the adrenals was increased after ovariectomy. Administration of testosterone propionate to castrated males led to a decrease in adrenal gland weight and to a pronounced increase in the succinoxidase activity, thus reversing the alterations induced by castration. Administration of estradiol dipropionate to ovariectomized females resulted in an increase in absolute and relative adrenal gland weight and in an increase in succinoxidase activity of the glands. Progesterone exerted no effect.

The oxygen consumption of adrenal gland slices from male rats, expressed per unit of fresh weight was higher than that of similar preparations from female animals in the presence of succinate or of glucose. The oxygen uptake per pair of sliced glands, however, was not different in the two sexes.

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THE INFLUENCE OF PROGESTERONE, RELAXIN AND ESTROGEN ON SOME STRUCTURAL AND FUNCTIONAL CHANGES IN THE PRE-PARTURIENT MOUSE

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THE occurrence of parturition at the normal time in mice has been reported to depend upon the simultaneous presence of the ovaries and placentae (1, 2). Although progesterone is necessary for maintenance of normal intrauterine pregnancy in the absence of the ovaries in mice and certain other species (3, 4, 5, 6), it may delay or prevent parturition (7, 8, 9, 10) and thereby lead to fetal death (10, 11, 12, 13) even when treatment is stopped on the 19th or 20th day of pregnancy (3, 14, 15).

The pubic symphysis fails to open in spayed or testosterone-treated pregnant mice, and this phenomenon may be associated with parturient failure (2, 3, 16, 17). Bioassays of pregnant mouse tissues detect relaxin activity only in ovaries and serum, and not in placentae, uteri or kidneys (15), lending support to the thesis that ovariectomy deprives this species of the hormone. While Crelin (18) showed that mechanical prevention of symphyseal separation did not preclude uneventful parturition, his experiment does not deny a definite role of relaxin in the delivery mechanism. Recent reports indicate that relaxin extracts make possible delivery of living young to prepuberal or adult pregnant mice castrated and maintained on progesterone (3, 14), and that relaxin, in combination with progesterone, results in the successful maintenance of pregnancy and induction of labor and delivery with oxytocin in ovariectomized adult mice (17).

The present paper expands the latter finding and presents evidence that progesterone and relaxin have distinct effects on the mechanical and physiological aspects of parturition in the mouse.

MATERIALS AND METHODS

General: Pregnant albino mice (CFW strain, 8–12 weeks of age at breeding) were used in all experiments. Pregnancy was dated by mating large numbers of animals for a single night, isolating the females, and selecting the obviously pregnant mice early in the third week of gestation.

In order to characterize the normal events leading to, and following parturition, five or more intact pregnant mice were sacrificed daily from the 15th to 20th day of pregnancy, and 1, 2 and 5 days postpartum.

Experimental: On the 16th day of pregnancy, mice were either bilaterally or unilater-

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ally ovariectomized, sham operated, or left intact. Sham operation consisted of dorsolateral incisions, entrance into the abdominal cavity and section of the fallopian tubes. Progesterone (1 mg./0.1 ml. sesame oil) and estradiol-17β (0.2–125 μg./0.1 ml. Mazola oil) were injected on the 16th, 17th and 18th days of pregnancy. Relaxin extract made from swine ovaries[1] was administered subcutaneously on days 16 and 18 in 0.2 ml. 5% beeswax in peanut oil (20) at dosages equivalent to 3.3 and 6.7 mg. of our relaxin reference standard (19). Half of the control animals received only the vehicles.

On the morning of the 19th day of pregnancy, individually caged animals were injected with 0.5 I.U. oxytocin extract[2] in 0.2 ml. 20% polyvinylpyrrolidone (PVP) solution. This dose and vehicle were found in preliminary trials to be more effective in terms of prolonged, controlled labor. Times of births and conditions of fetuses were recorded. Observations were made for 4 hours after injection (oxytocic activity observed as externally apparent uterine contractions, was not evident in most cases after 2 to 3 hours). Mice were then sacrificed, and fetal weights, total number of fetuses in utero, cervical "dilatability"[3] and interpubic ligament lengths were recorded.

Cervical "dilatability" was measured by passage of a tapered lucite rod, graduated in 0.5 mm. through the cervical canal (from the external os) until firm resistance was felt, after the method described by DeVaal (21). The symphysis pubis was exposed and the interpubic ligament measured directly.

In order to quantitate data, the following calculations were made:

$$\text{A. } \textit{Average Percentage of Each Litter Delivered} = \frac{\text{No. of fetuses delivered}}{\text{Total fetuses (delivered and in utero)}} \times 100$$

Average percentage of each litter delivered was selected as the most reliable criterion of responsiveness of the delivery mechanism to oxytocin. This is a more critical measurement than "*percentage of mothers delivered,*" as it is not appreciably influenced by mice which give birth to only 1 or 2 fetuses. All mice in a group are included in this calculation.

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[1] Releasin®, Warner-Chilcott, preparation #W1164A—Lot 5–7.

[2] Pitocin®, Parke, Davis.

[3] Measurement of cervical dilatability in 19-day pregnant mice sacrificed before, and four hours after, oxytocin treatment revealed the following:

Treatment	Before oxytocin		After oxytocin	
	No. mice	Cervical dilatability mm.	No. mice	Cervical dilatability mm.
None	10	5.7 ±0.31	15	5.5 ±0.34
Unilateral ovariex	6	6.5 ±0.37	13	6.4 ±0.26
Bilateral ovariex (progesterone)	13	3.4 ±0.33	12	5.0 ±0.59

The increased cervical dilatability observed following induced labor in bilaterally ovariectomized, progesterone maintained mice was statistically significant ($P < 0.05$, > 0.02). The *biological* significance may be questioned, pending further investigation, as, following labor, the cervices of these mice were similar in dilatability to those of intact mice, although in most instances delivery had *not* occurred. As no increase in dilatability was observed following oxytocin treatment of intact or unilaterally ovariectomized pregnant mice, it was concluded that, for the purposes of this study, it was justifiable to measure cervical dilatability following labor. It was further decided that significance of observations made on the cervix should be tested at the 99% confidence level, since the technique is both crude and subjective.

B. *Latent Period = time between oxytocin injection and birth of the first fetus.*

Oxytocin activity begins soon after injection as evidenced by visible contractions through the abdominal wall, opisthotonos and general discomfort.

The latent period measures the speed with which this activity progresses to effective labor.

C. *Fetal Birth Rate = Fetuses/hour/mother.*

Calculated from the time between the delivery of the first and last fetus. It could be argued that this calculation should include the time between *injection* and the birth of the last fetus, but this would bias the data in favor of the latent period, which we have used as a separate measurement.

Fetal birth rate and latent period data only include animals which actually delivered. It would have been possible to average in the latent periods of animals which did *not* deliver as " >4 hours," but such figures would not agree with those obtained from mice in the same groups which *did* deliver, suggesting that *failure* to deliver in 4 hours represents something other than a *prolonged* latent period.

Upon analysis of the data it was discovered that in a few litters fetal weights were extremely low and did not appear to be congruous with the majority of animals. There were also mice with a litter size of less than 5 fetuses. While there was no indication of any difference in the responsiveness of such animals to the various treatments, mice with less than 5 total fetuses or with fetuses weighing less than 0.4 gm. each were excluded from the data for the sake of uniformity.[4]

All critical experiments were repeated at least three times with small numbers in parallel with appropriate controls and the data to be presented represents a pooling of such groups.

RESULTS

Normal pregnancy changes. Figure 1 illustrates the normal course of separation of the pubes (confirming by direct measurement the work of Hall and Newton (22)), increased cervical dilatability and enhanced responsiveness to oxytocin from the 15th day of pregnancy until term. This is consistent with findings in other species (6, 21, 23, 24 25). Following parturition, (which occurred between the 20th and 22nd day p.c.), there was observed a rapid regression of the interpubic ligament and cervix, which approached pre-pregnant dimensions about 5 days postpartum.

The similarity of the three curves suggested a possible common hormonal relationship, and accordingly the following experimental work was undertaken.

Influence of Operation. Intact, untreated CFW mice were observed to deliver spontaneously on the 20–22nd day of gestation. Mice totally ovariectomized on day 16 aborted within 24 hours unless treated immedi-

[4] A total of 13 mice was excluded from 9 different groups. There was no apparent correlation between these abnormalities and any of the hormonal treatments employed. Reduced litter size was either due to unilateral pregnancy or partial abortion caused by operative trauma. Three of 15 sham operated untreated mice aborted during these and other studies. Reduced fetal weight may have been due to delayed implantation, as these fetuses were normal and similar to those taken from other mothers on the 17th day-of-pregnancy.

ately with 1 mg. progesterone. Depot relaxin or a combination of depot and saline solution of relaxin did not duplicate this progesterone effect. Unilateral ovariectomy did not precipitate abortion. This treatment did, however, significantly increase the percentage of each litter delivered with oxytocin on day 19 of pregnancy from 33% (sham operated) to 92% ($P < 0.01$; Table 1; Fig. 2).

Influence of Progesterone. Injection of 1 mg. progesterone daily, days 16–18 of pregnancy in intact and unilaterally ovariectomized mice, sharply reduced the percentage of births induced with oxytocin on the 19th day of

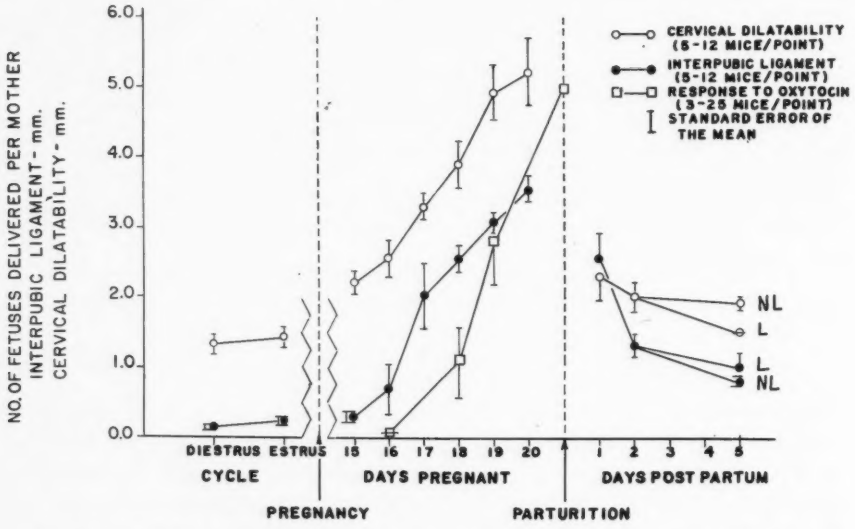

FIG. 1. Normal changes in interpubic ligament length and cervical dilatability in the cycling, pregnant and postpartum mouse, and increased oxytocin responsiveness with advancing pregnancy. L = lactating. NL = not lactating.

gestation. However, progesterone treated unilaterally operated animals were nearly similar to intact, untreated mice in their low order of responsiveness (Fig. 2).

Bilaterally ovariectomized mice maintained on progesterone were also unresponsive to oxytocin injection, and in addition, the interpubic ligaments of these animals failed to elongate (Fig. 2, Table 1).

The cervical canal showed marked dilatability on the 19th day of gestation in all groups, and the interpubic ligaments of intact and unilaterally ovariectomized mice increased significantly in length despite progesterone administration.

Progesterone treatment tended to increase the latent period in all groups (Table 1).

TABLE 1. INFLUENCE OF OVARIECTOMY AND HORMONAL THERAPY ON INTERPUBIC LIGAMENT FORMATION, CERVICAL DILATABILITY AND DELIVERY RESPONSE TO OXYTOCIN IN 19-DAY PREGNANT MICE

Group	Operation	Treatment	No. mice	Fetuses delivered		Latent period time of first fetus, minutes	Fetal birth rate, fetuses/hour	Cervical dilatability, mm.	Interpubic ligament, mm.
				Per mother	Average % of each litter				
I	none	none (or +placebo)	15	2.6±0.6*	30± 7.4	58± 9	3.1±0.56	5.5±0.34	3.14±0.26
II	sham op.	none (or +placebo)	10	3.0±1.0	33±10.8	62±10	2.6±0.57	5.4±0.38	3.79±0.33
III	none	progesterone, 1 mg. days 16, 17, 18	10	0.1±0.1	1± 1.0	84	—	4.6±0.31	2.14±0.28
IV	none	relaxin, 6.7 mg. days 16 & 18	9	4.1±1.2	41±12.5	49± 6	3.3±0.66	6.2±0.41	4.95±0.39
V	none	progesterone, 1 mg. days 16, 17, 18+relaxin, 6.7 mg. days 16 & 18	9	1.8±0.7	19± 7.6	72±12	2.9±0.46	5.4±0.35	4.35±0.29
VI	none	progesterone, 1 mg. days 16, 17, 18+estradiol-17β, 1, 25 or 125 μg. days 16, 17, 18	19	1.3±0.5	11± 5.0	119±38	4.1±0.94	5.4±0.28	2.94±0.19
VII	none	estradiol-17β, 25 μg. days 16, 17, 18	11	3.0±1.1	40±14.0	53± 9	8.2±2.46	4.5±0.28	3.68±0.22
VIII	none	estradiol-17β, 25 μg. days 16, 17, 18+relaxin, 6.7 mg. days 16 & 18	10	5.2±1.3	66±10.5	56±21	5.4±1.23	5.1±0.33	4.62±0.44
IX	none	estradiol-17β, 25 μg. +progesterone, 1 mg. days 16, 17, 18+relaxin, 6.7 mg. days 16 & 18	8	2.1±0.6	27± 8.0	61±10	2.6±0.38	4.9±0.38	3.33±0.49
X	½ ovariex	none	13	8.5±0.6	92± 5.3	46±11	6.9±0.69	6.4±0.26	3.41±0.19
XI	½ ovariex	progesterone, 1 mg. days 16, 17, 18	8	1.9±0.8	20± 8.4	66±18	3.7±1.00	6.0±0.74	3.19±0.43
XII	½ ovariex	relaxin, 6.7 mg. days 16 & 18	8	9.0±0.7	91± 4.4	42± 6	5.9±1.10	6.6±0.40	5.11±0.28
XIII	½ ovariex	progesterone, 1 mg. days 16, 17, 18+relaxin, 6.7 mg. days 16 & 18	10	2.4±0.7	34±11.0	48± 7	5.1±1.55	5.4±0.56	4.05±0.27
XIV	ovariex	progesterone, 1 mg. days 16, 17, 18	12	1.0±0.7	12± 8.0	88	1.9	5.0±0.59	0.81±0.12
XV	ovariex	progesterone, 1 mg. days 16, 17, 18+relaxin, 6.7 mg. days 16 & 18	11	5.8±0.8	69±10.6	33± 5	2.7±0.70	7.4±0.25	3.94±0.16
XVI	ovariex	progesterone, 1 mg. days 16, 17, 18+estradiol-17β, 0.2, 1.0, 5.0, 25.0 or 125 μg. days 16, 17, 18	28	0.7±0.3	8± 3.4	124±19	1.5±0.28	4.5±0.32	0.67±0.06
			4	0	0	—	—	2.9±0.39	0.88±0.31
A	ovariex	progesterone, 1 mg. days 16, 17, 18+0.2 μg. E	10	0.9±0.5	10± 4.8	132±32	1.2	5.3±0.61	0.62±0.16
B	ovariex	progesterone, 1 mg. days 16, 17, 18+1.0 μg. E	5	0	0	—	—	3.6±1.00	0.57±0.10
C	ovariex	progesterone, 1 mg. days 16, 17, 18+5.0 μg. E	4	2.0±2.0	20±20.0	93	2.3	5.2±1.33	0.81±0.22
D	ovariex	progesterone, 1 mg. days 16, 17, 18+25.0 μg. E	5	0.6±0.4	6± 4.0	156	1.3	5.0±0.55	0.78±0.12
E	ovariex	progesterone, 1 mg. days 16, 17, 18+125.0 μg. E							

* Standard error of the mean.

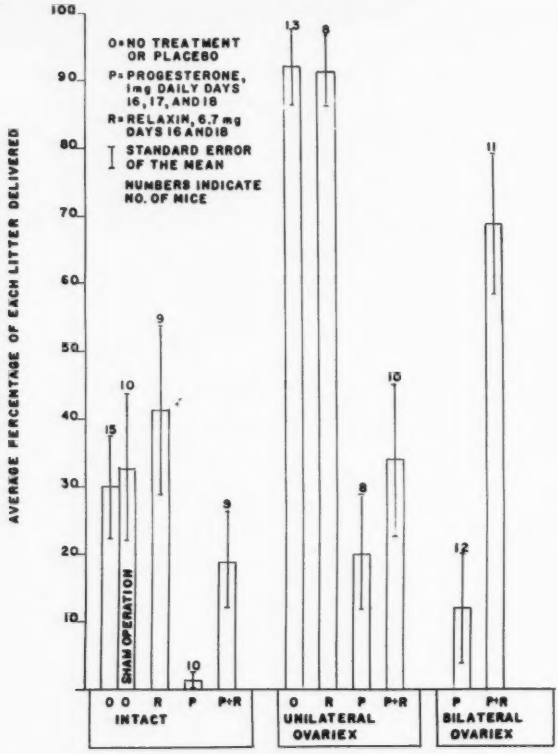

FIG. 2. Influence of ovariectomy, progesterone and relaxin on delivery response to oxytocin injection on the 19th day of pregnancy in mice.

Influence of Relaxin. Injection of 3.3 or 6.7 mg. of relaxin extract in intact or unilaterally ovariectomized pregnant mice did not significantly alter their response to oxytocin on day 19 of pregnancy. When progesterone was concomitantly administered, a result was obtained which was intermediate between no treatment and that observed with progesterone alone (Fig. 2).

In totally ovariectomized mice maintained on progesterone, relaxin injection increased the percentage of each litter delivered from 12 to 69. The latent period between time of oxytocin injection and appearance of the first fetus was significantly shorter in bilaterally ovariectomized mice maintained on relaxin and progesterone than in intact animals in which labor was similarly induced.

Whereas progesterone treatment of intact mice had suppressed elongation of the interpubic ligament (2.14 mm. vs. 3.14 mm. in intact controls; $P < 0.02$), relaxin injections, alone or in combination with progesterone, increased the symphyseal separation to well over 4 mm. ($P < 0.01$). In

unilaterally ovariectomized animals, the mean interpubic ligament length was 3.41 mm. Relaxin treatment of such mice induced a separation of the pubes averaging 5.11 mm. ($P < 0.01$). Concomitant progesterone therapy reduced this effect (4.05 mm; $P < 0.02$). Interpubic ligaments of progesterone-maintained, bilaterally ovariectomized animals measured only 0.81 mm., whereas a mean pubic separation of 3.94 mm. was observed in mice treated with relaxin in addition to the progesterone.

Relaxin had no consistent effect on dilatability of the cervix on the 19th

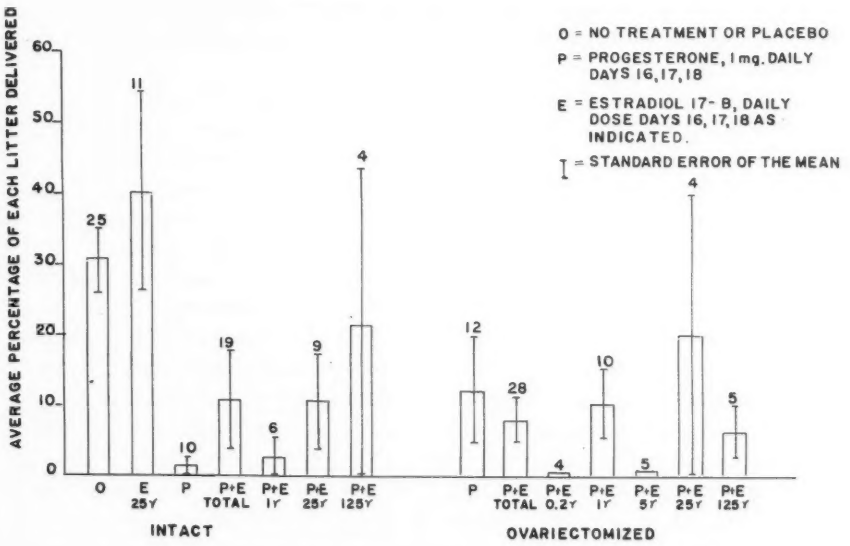

FIG. 3. Influence of progesterone and estrogen on delivery response to oxytocin injection on the 19th day-of-pregnancy in mice.

day of pregnancy. However, it was observed that in totally ovariectomized mice, the cervix was generally more dilatable when a combination of relaxin and progesterone was injected, than when progesterone was the only therapy used (7.4 vs. 5.0 mm.; $P < 0.01$).

Influence of Estrogen. Injection of 25 μg. estradiol-17β, days 16, 17 and 18 of pregnancy, in intact mice did not influence response to oxytocin on day 19, alter pelvic changes or affect cervical dilatability (Fig. 3, Table 1). In intact, progesterone treated mice, 1, 25 or 125 μg. of estradiol-17β increased the number of mothers delivering and tended to increase the percentage of litters delivered (not significant at $P = .05$), although the increase was still significantly below nontreated control values. Estrogen-progesterone treated mice exhibited extremely long latent periods following oxytocin injection. In ovariectomized mice, injection of estradiol-

17β (0.2, 1.0, 5.0, 25.0 or 125 μg.) did not appear to modify the effects of progesterone. Estrogen may have augmented relaxin effects, as injection of 25 μg. estradiol-17β into intact animals treated concomitantly with relaxin, or with progesterone and relaxin, further increased responsiveness to oxytocin, although not significantly at the 95% confidence level (Table 1). Estrogen in any of the dosages failed to induce pubic separation in totally spayed mice.

DISCUSSION

These results strongly suggest the existence of a synergism between relaxin and progesterone at the uterine and pelvic levels during the latter stages of pregnancy in the mouse.

The intact untreated mouse is relatively resistant to oxytocin on the 19th day of gestation in terms of average percentage of each litter delivered. Removal of one ovary greatly increases the responsiveness, presumably by reducing endogenous progestational hormone levels, while not significantly decreasing the activity of a relaxative substance. This is the most plausible explanation, since injection of progesterone (at a physiological level) into unilaterally ovariectomized animals only reduced responsiveness to oxytocin to slightly below the level of intact, untreated mice; whereas, progesterone treatment of intact mice reduced responsiveness to nearly zero. It is suggested that progesterone may prolong pregnancy by this mechanism. Relaxin injection in intact mice treated with progesterone significantly increased the percentage of each litter delivered with oxytocin when compared with appropriate controls. Totally ovariectomized mice maintained on progesterone were generally unresponsive to oxytocin and failed to show separation of the pubes. Concomitant relaxin injection completely reversed this picture, effecting symphyseal relaxation and rendering animals highly responsive to the posterior pituitary hormone.

These data are interpreted to indicate that responsiveness to oxytocin and pelvic changes in preparation for parturition in the mouse are dependent upon a delicate balance of relaxin and progesterone. A hormonal shift in favor of relaxin increases responsiveness to oxytocin and interpubic ligament formation, whereas excessive progesterone decreases effectiveness of oxytocin and partially blocks pubic separation.

Estradiol-17β at the dosage levels tested did not significantly affect responsiveness to ocytocin, nor did it induce interpubic ligament formation in spayed mice. Exogenous estrogen was not necessary for the action of relaxin. This is in accord with the observations of Smithberg and Runner (3).

This work confirms in the mouse the progesterone "block" described by Csapo (6), but does not support the thesis that a drop in progesterone levels is an initiating factor in the parturient process. When unilateral

ovariectomy was performed on the 16th day of pregnancy, gestation continued despite the marked increase in the responsiveness of the delivery mechanism to exogenous oxytocin. Similarly, although relaxin treatment *increased* oxytocin responsiveness of bilaterally ovariectomized animals maintained by progesterone injection, parturition did not occur. Thus, it is possible to have a receptive delivery mechanism in the absence of parturition. This can be achieved by decreasing progesterone or increasing relaxin levels. Further, the presumed inhibitory effect of progesterone on uterine motility and responsiveness to oxytocin reported by many investigators (6, 26, 27, 28, 29) and contested by others (30, 31, 32, 33, 34) must be reconsidered in the light of present evidence which demonstrates that a progesterone-dominated uterus can be induced to expel its contents effectively if sufficient relaxin is concomitantly present.

Hormonal factors essential for dilatation of the cervix in the mouse have not been elucidated. Relaxin softens the cervix in the non-pregnant gilt (35), cow (36), human (37), rat and mouse (15). However, cervical softening progressed in ovariectomized pregnant mice maintained on progesterone alone despite the failure of the symphysis pubis to open. Thus, in this species, progesterone, estrogen, or some other hormonal or mechanical factor must therefore be capable of producing significant changes in the cervix of pregnancy, not withstanding our unpublished evidence that relaxin injection can produce such changes in the nonpregnant mouse.

Similar changes have been described in the cervix of the pregnant rat (21, 24, 25). There is evidence that this increased dilatability is hormonally regulated (24) and a role of relaxin is inferred (25).

SUMMARY

Some normal physiological and anatomical adaptations preceding and following parturition in the mouse were described. The work of Hall and Newton (22) was confirmed by direct measurement technique.

Unilateral ovariectomy on day 16 of pregnancy did not influence cervical softening or symphyseal separation. It did bring about a highly significant increase in responsiveness to oxytocin of the delivery mechanism on the 19th day of pregnancy.

Progesterone treatment decreased responsiveness to oxytocin injection of the 19th day of pregnancy in intact, unilaterally or bilaterally ovariectomized pregnant mice.

Pretreatment with relaxin markedly increased responsiveness to oxytocin and induced interpubic ligament formation in bilaterally ovariectomized progesterone-maintained mice. Estradiol-17β over a wide dosage range did not duplicate these effects.

Cervical softening progressed in the absence of ovarian tissue in progesterone-maintained pregnant mice.

Acknowledgment

Gratitude is expressed to Mr. P. L. Nemith for technical assistance.

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PLASMA LEVELS OF PROGESTIN DURING PREGNANCY IN THE MOUSE[1]

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THUS far, the compound progesterone has apparently been identified in peripheral blood during pregnancy only in the human being, and there in quite low concentrations (1, 2, 3). Bioassay, on the other hand, has revealed biologic activity, attributed earlier solely to progesterone, in peripheral blood of several species during pregnancy. If, as seems probable, the biologically active material is the circulating form or forms of the luteal hormone (here referred to as progestin), it is at least as important to know when during pregnancy the material is present and when changes in level occur as it is to know the chemical identity of the active materials. The present paper describes an attempt to obtain these data for the mouse.

MATERIALS AND METHODS

The mice studied were members of the A strain of Strong. Females in breeding cages were examined each morning, and pregnancies were dated from the day a vaginal plug was discovered. Blood samples were obtained by cardiac puncture or by severing a carotid artery under ether anesthesia. In each instance the blood (0.5 to 1.0 ml.) was collected in a syringe or tube containing 0.05 ml. 20% sodium citrate solution. Each animal served once as a donor. Pregnancies were verified by gross inspection of the uterus or, during the first four days, by finding ova or dividing ova in serially sectioned uterine tubes. Fifty-three samples from as many mice known to be pregnant were obtained.

The citrated blood was centrifuged within minutes after being drawn, and the measured plasma was immediately frozen for later assay, or added to cold acetone for separation of the "free" and "bound" fractions (4). Assays were conducted according to our standard procedure (5). It has been shown that identical values are obtained upon assay of cold acetone extracts and of frozen raw plasma from the same sample of blood (4).

The ovaries of 23 donors of blood samples were fixed in Bouin's fluid, sectioned serially at 6μ, and stained with hematoxylin and eosin.

RESULTS

The assays are expressed in Progesterone Equivalents (P.E.); one P.E. has been defined (6) as *activity* equal to that of 1.0 μg. of progesterone when assayed by the method of Hooker and Forbes in the absence of estrogen or other interfering factors.

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The values given by the assays of both "free" and "bound" fractions are shown in Figure 1. It is to be emphasized that each value for "free hormone" represents a different animal and that each value for "bound hormone" represents a different animal, although both "free hormone" and "bound hormone" for the same animal are shown in most instances.

The values for "free hormone" varied widely from animal to animal during the first three days of pregnancy. Thereafter, the values for different animals on the same day of pregnancy corresponded quite well.

From day 4 until day 7 the values for "free hormone" increased rapidly. From day 7 until day 9 a high level was maintained. From day 9 to day 12

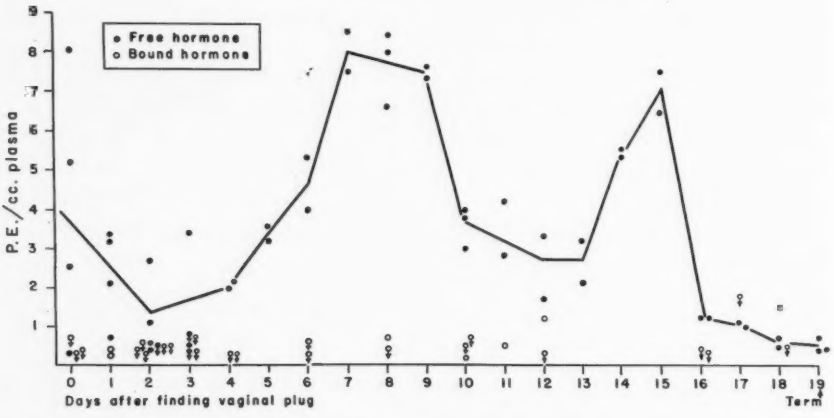

FIG. 1. "Free" and "bound" levels of progestin during pregnancy as revealed by bio-assay of systemic plasma. Symbols with arrows indicate that the value is below the concentration indicated by the symbol.

the level declined, at first rapidly and then more gradually. From day 13 to day 15 the level again rose rapidly, and had fallen sharply by the next day. The levels were relatively quite low on days 16 to 19. Three mice served as donors on day 19. One was bled before delivery had begun, one during delivery, and one between two and five and one-half hours after delivery.

The values for "bound hormone" were consistently low, being with three exceptions less than 0.8 P.E./ml. plasma. The three exceptions were samples from animals 12, 17, and 18 days pregnant.

One or both ovaries from one to three animals bled on each of days 0, 1, 3, 5, 7, 9, 11, 13, 14, 15, 17, and 19 were studied microscopically, with most attention being given the corpora lutea. In all of the animals bled on day 0 through day 13 and in one animal bled on day 14 the luteal cells exhibited a clear or somewhat foamy cytoplasm. In a second animal bled on day 14 and in all of the animals bled later in pregnancy the luteal cells

were characterized by granular cytoplasm. In two of the animals bled on day 13 and in all animals bled later in pregnancy the cytoplasm of the luteal cells contained circumscribed, strongly eosinophilic, juxtanuclear areas.

DISCUSSION

The great scattering of the values for "free hormone" on day 0 and the lesser scattering on days 1, 2, and 3 admit of no confident explanation. An obvious possibility is inaccurate estimation of the exact duration of pregnancy. The hour of mating itself was not known, and it was impracticable to draw all samples at the same hour. It is also possible that in early pregnancy the level of "free hormone" fluctuates rapidly and conspicuously. Such fluctuations have been found in rabbit's blood obtained at hourly intervals before and after ovulation (7). The consistency in the values after day 3 bespeaks the reproducibility of the assays and the reduction in biologic variability gained by use of inbred mice.

It may be assumed that the elevation in values to day 7 and the maintenance of a high level through day 9 reflect increasing and sustained activity of the corpora lutea. At any rate, no major non-luteal source of progesterone during this portion of pregnancy has been reported for the mouse. If the assumption is correct, it follows that the decline in values after day 9 probably reflects diminished luteal activity. Interestingly, the decline in blood values for "free hormone" antedated by three days cytologic changes in the corpora lutea that can be considered regressive.

It seems reasonable to suppose that the placenta is a site of origin of the progestational material that reached a high level on day 15. It will be recalled that progestational activity has been found in placental extracts and that progesterone has been isolated from human and mare placentae (2, 8, 9, 10, 11). If the activity in plasma after day 13 is at least partially placental in origin, it seems equally reasonable to suppose that the material revealed by bioassay on days 12, 13, and 14 may have its origin in both placentae and corpora lutea. In any case, projections of the descending curve after the first peak and of the ascending curve before the second peak (Fig. 1) intersect this portion of the abscissa.

The sharp decline in assay values after the second peak preceded parturition by about four days, a considerable period of time in a pregnancy lasting but 19 days. This circumstance hardly supports the supposition that labor is at least partially precipitated by a decline in circulating progestin. On day sixteen the activities found were equivalent to but 1.2 μg. progesterone per ml. of plasma. It has been reported (12) that 1.0 μg. per ml. is the lowest plasma level that provokes histologically recognizable response in the endometrium of the ovariectomized mouse.

The suggestions above with respect to the assays and their fluctuations have been predicated upon the assumption that blood levels reflect levels

of secretion. So far as is now known, however, it is equally conceivable that fluctuations in plasma levels reflect fluctuations in the rate of inactivation of the active material, or that they reflect entry into and release from depots of storage. Irrespective of which, if any, of these possibilities is correct, the plasma level probably reflects the humoral environment of the pregnant uterus.

The values for "free hormone" during the first twelve days of pregnancy for the most part correspond quite well with the intensity of the response to progestin exhibited by the endometrium during the same period in the same strain of mice (13). The peak during later pregnancy, however, contrasts with the poor response to progestin shown by the endometrium (13). These circumstances suggest that during the first twelve days the morphologic character of the endometrium reflects promptly and reasonably accurately the level of progestin to which it is exposed. They also suggest that from day 12 to day 19 the endometrium responds little to progestin in its environment. In harmony with the latter suggestion is the observation of Atkinson (14) that the non-pregnant uterine cornua of unilaterally pregnant mice do not form deciduomata after the tenth day of gestation, even if progesterone is injected. The failure to respond to usually effective blood levels of progestin may offer an important clue to various problems inherent in the latter portion of pregnancy. Antagonism of the blood progestin by estrogen does not seem an adequate explanation, inasmuch as an earlier study has shown that the endometrium does not in later pregnancy give evidence of intense stimulation by estrogen (13).

The curve of "free hormone" through pregnancy in the mouse is unlike those for other species obtained by the same assay technique. In the ewe (15) and in the rabbit (16) progestin levels rose more or less progressively, while in the human being (17, 18) and in the monkey (17) virtually cyclic fluctuations were found with no really high levels at any time. No consistent relationship exists between progestin levels at various stages in pregnancy and the effect of ovariectomy upon the continuation of pregnancy. In the rabbit (19) and in the mouse (20, 21) ovariectomy interrupts pregnancy at all stages, although the curves of blood levels of progestin through pregnancy are quite different in the two species. Similarly, with equally different curves of blood levels of progestin through pregnancy, ovariectomy is followed by abortion only in early pregnancy in the human being (22, 23), the monkey (24) and the ewe (25, 15). In this connection it is interesting that ovariectomy is followed by a prompt fall of blood progestin and by abortion in the rabbit (16), and by abortion without a decrease in blood progestin in the ewe (15).

The blood level at parturition is equally inconsistent from species to species. High levels are present at this time in the ewe (15) and in the rabbit (16), whereas a sharp decline antedated parturition by several days in the mouse. In the human being and in the monkey the blood level of

progestin at parturition varied from individual to individual, with no consistent pattern evident (17). Accordingly, the relationship, if there is one, of progestin to the onset of labor remains obscure.

The identity of the material assessed by the bioassay is uncertain. Some 32 compounds that are similar to progesterone or are possible precursors, metabolites, or contaminants of progesterone have failed, except in exceedingly large amounts, to elicit the reaction upon which the assay depends (5, 26, 27). Recently two compounds that appear to be metabolites of progesterone have been found, like progesterone, to elicit the reaction (28); they are Δ^4-3-ketopregnene-20α-ol and Δ^4-3-ketopregnene-20β-ol. The relative amounts of these and other compounds which may be present in the biologically active progestin in the blood must be determined by further studies.

SUMMARY

Fifty-three mated female mice of the A strain of Strong were bled on days 0 through 19 after the finding of a vaginal plug. The existence of pregnancy was verified in the two to four mice bled on each of the 20 days. A plasma sample from each animal was subjected to a bio-assay which measures collectively progesterone, Δ^4-3-ketopregnene-20α-ol, and Δ^4-3-ketopregnene-20β-ol. The results, expressed in Progesterone Equivalents (P.E.), were plotted against time. "Bound" levels of progestin seldom exceeded 0.7 P.E./ml. The "free" progestin level rose to a peak of about 8 P.E./ml. on the 7th and 8th days, fell sharply to about 3 P.E./ml. on the 12th and 13th days, rose again to a peak of approximately 7 P.E./ml. on the 15th day, fell sharply to 1.2 P.E./ml. between the 15th and 16th days, and diminished thereafter until delivery of the young on the 19th day. Histological study of the corpora lutea suggested that their period of activity is maintained until just after the first peak of plasma progestin occurs (i.e., until about day 14).

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CHANGES IN THE COMPOSITION OF THE RAT UTERUS FOLLOWING A SINGLE INJECTION OF RELAXIN

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THE effects of estrogen and progesterone on uterine composition have been under investigation for some time (for review and discussion see Reynolds, 1). Whereas relaxin has been shown to inhibit uterine motility *in vivo* and *in vitro* (2, 3), to modify the histology and increase dilatability of the uterine cervix (4, 5) and very recently, to increase uterine water content (6), its effects on other aspects of uterine physiology and biochemistry have not been elucidated.

The present report concerns the effects of a single low dose of relaxin on uterine glycogen, water and nitrogen in the estrogen primed spayed rat.

MATERIALS AND METHODS

White rats of an inbred commercial strain (Budd Mountain) were used in all experiments. The rats were spayed when 30–32 days old and estrogen primed two weeks later. A small priming dose of estrogen was found to be necessary for marked relaxin effects. The estrogen dose chosen did not increase uterine glycogen concentration over castrate control values, and only increased water content slightly (81.5 vs. 80.0%). Estrogen priming consisted of a single subcutaneous injection of 5 μg. estradiol-cyclopentylpropionate[2] (ECP) in 0.1 ml. peanut oil on day one. On day eight, the rats received subcutaneously 0.1 mg. of our relaxin reference standard[3] (7) in 0.2 ml. 1% L-390[4] (benzopurpurine 4-B), an azo dye which has been found to potentiate relaxin in mice and rats, presumably by retarding absorption of the hormone from the site of injection. Controls received only the vehicle. Preliminary studies had shown that with L-390 the relaxin dose selected produced a 75–100% increase in uterine glycogen at the optimal time post-injection for this species. In other experiments, relaxin was administered in PVP, beeswax-oil and saline solution. Rats were sacrificed at 3, 6, 12, 24 and 48 hours following the relaxin dose. Parallel controls were autopsied at identical times after injection of the vehicle alone. In order to test specificity of the effects, a low potent relaxin extract[5] was injected at equivalent mg. and potency concentrations in comparison with the standard.

Uteri were split through the cervix, blotted, weighed, and one horn was fast frozen on

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[1] Present address: Dept. of Zoology, Rutgers University, New Brunswick, New Jersey.

[2] Depo®-estradiol, Upjohn.

[3] Releasin preparation W1164-A Lot 8, assaying 150 units/mg. by guinea pig symphysis pubis palpation test.

[4] Kindly furnished by Dr. Robert Sanders and Mr. R. J. Palazzolo, Lambert-Hudnut Research, Div. of Warner-Lambert Pharmaceutical Company.

[5] Preparation W1164-A Lot 36; each mg. equivalent in potency to 0.07 mg. relaxin reference standard.

dry ice for glycogen estimation. The other horn was placed on aluminum foil for dry weight determination. Control studies had demonstrated no significant differences in glycogen and water concentrations of the two horns.

Glycogen was precipitated and purified according to the method of Walaas (8) and estimated by the anthrone procedure of Seifter *et al.* (9).

Water content was calculated after drying the tissues in a vacuum oven at 60° C. for a minimum of $3\frac{1}{2}$–4 hours. As only one-half of the uterus was used for determination of water concentration, *total* uterine dry weight was derived by calculation, employing the total uterine wet weight and percentage water. Nitrogen concentration of pooled dried tissues of each group was estimated by a standard microKjeldahl method, and total nitrogen per uterus calculated on the basis of average uterine dry weight.

RESULTS

The water content of uteri of relaxin treated rats rose rapidly reaching a maximum of 85.4% in 6 hours and then dropped to 82.9% in 24 hours, reaching control levels of 81.5% in 48 hours. Uterine weight increase paralleled the water uptake, reaching a maximum value in six hours. Nitrogen determinations and uterine dry weights indicated nitrogen deposition occurred between 0 and 12 hours (Table 1 and Fig. 1), although nitrogen *concentration* decreased due to the increased hydration (Table 1).

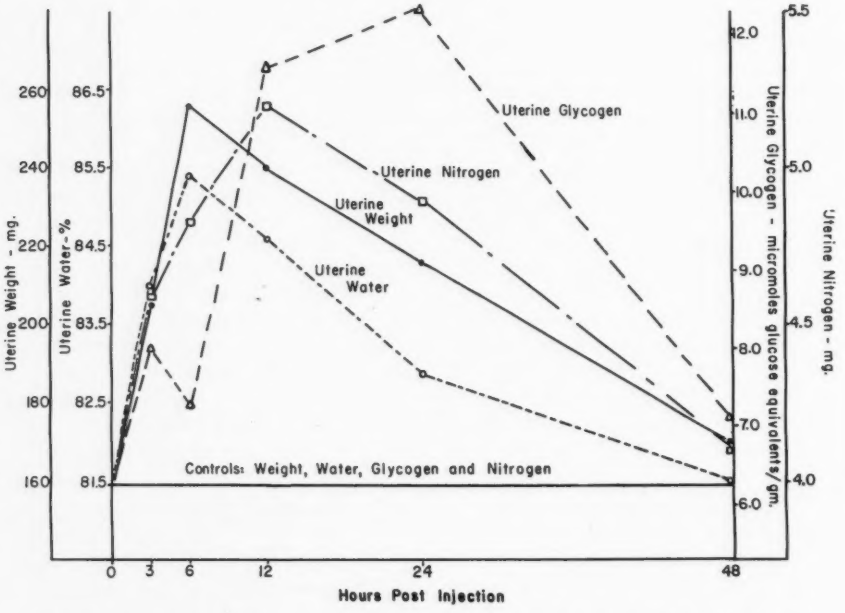

FIG. 1. Changes in uterine composition following a single injection of relaxin in estrogen primed spayed rats. Experimental rats received 0.1 mg. relaxin reference standard in 0.2 ml. 1% L-390. Controls received only the vehicle.

Uterine glycogen concentration increased from 6 to nearly 12 micromoles glucose equivalents/gm. fresh tissue in 12 hours and plateaued for an additional 12 hour period before dropping to control levels in 48 hours.

When the relaxin extract was administered in saline solution or depoted in other vehicles, qualitatively similar effects were observed, but the time relationships varied. PVP was as active a vehicle as L-390, while relaxin in 5% beeswax in peanut oil or saline solution induced a lesser rise in uterine glycogen, although uterine water was as markedly increased (Table 2).

A good comparison between standard and low potent preparation Lot

TABLE 1. CHANGES IN UTERINE WEIGHT, WATER, GLYCOGEN AND NITROGEN FOLLOWING A SINGLE INJECTION OF RELAXIN IN SPAYED, ESTROGEN PRIMED RATS

Treatment*	Time between injection and sacrifice	No. rats	Uterine wet weight, mg	Uterine dry weight, mg.	Uterine glycogen micromoles glucose, equivalents/gm.	Uterine water %	Uterine nitrogen, mg.	Uterine nitrogen, % wet weight
Placebo	3 Hours	4	161.0± 6.6†	29.0±2.4	5.9±0.4	82.0±0.24	3.96	2.46
0.1 mg. relaxin Std.		8	215.3± 6.9	34.3±1.6	8.0±0.3	84.0±0.29	4.58	2.13
Placebo	6 Hours	7	164.4± 7.1	30.4±1.6	7.2±0.6	81.5±0.24	4.04	2.46
0.1 mg. relaxin Std.		8	256.5±10.4	37.5±1.0	7.3±0.5	85.4±0.27	4.84	1.88
Placebo	12 Hours	7	155.5± 8.3	28.8±1.6	5.7±0.9	81.4±0.09	3.90	2.51
0.1 mg. relaxin Std.		9	239.5±13.7	37.0±2.4	11.6±0.9	84.6±0.24	5.20	2.17
Placebo	24 Hours	8	171.9± 6.3	32.0±1.0	7.0±0.7	81.4±0.12	4.15	2.42
0.1 mg. relaxin Std.		9	216.2± 8.0	36.7±1.4	12.4±0.9	82.9±0.26	4.89	2.26
Placebo	48 Hours	3	141.1± 5.6	26.8±0.9	4.1±0.2	81.0±0.17	3.49	2.48
0.1 mg. relaxin Std.		4	171.0± 6.3	31.9±2.6	7.1±0.5	81.4±0.15	4.15	2.43

* All rats were ovariectomized 2 weeks before use. All rats received 5 μg. ECP 8 days before these injections. Relaxin was injected in 0.2 ml. 1% L-390. Controls were injected with 0.2 ml. 1% L-390 as a placebo.
† Standard error of the mean.

36 was not obtained due to the shallowness of the dose-response curves. However, Lot 36 was significantly *less* potent than the standard when injected at an equivalent mg. concentration, and approximately as active on a relative potency basis (Table 3).

DISCUSSION

The rapidity of the glycogen and water increases, and the observed nitrogen deposition raise fundamental questions in regard to uterine physiology: namely, are the effects usually ascribed to estrogen in reality due to the transient formation of relaxin-like substances by the uterus itself? Or does relaxin either potentiate estrogen or make the uterus more receptive to the sex hormone? Boettiger (10), in discussing Astwood's (11, 12) and his own data, suggests that neither secretion of estrogen alone nor in combination with progesterone can account for the rapid changes in uterine

TABLE 2. INFLUENCE OF VEHICLE ON RELAXIN-INDUCED CHANGES IN COMPOSITION OF THE UTERUS OF ESTROGEN-PRIMED SPAYED RATS

Treatment* 0.1 mg. relaxin standard in:	No. rats	Time autopsied after injection									
		3 hours		6 hours		12 hours		24 hours		48 hours	
		Glycogen (glucose equiv.) μmoles/gm.	Water %	Glycogen (glucose equiv.) μmoles/gm.	Water %	Glycogen (glucose equiv.) μmoles/gm.	Water %	Glycogen (glucose equiv.) μmoles/gm.	Water %	Glycogen (glucose equiv.) μmoles/gm.	Water %
0.2 ml. Saline	17†	5.1±0.6	85.1± 0.5	7.5±1.5	84.0± 0.5	7.5±0.7	83.2±0.13	9.4±0.9	82.6±0.42	—	—
0.2 ml. 20% PVP	20†	6.0±0.2	85.1±0.17	7.6±1.0	85.5±0.67	8.9±0.5	83.1±0.26	12.8±1.8	82.1±0.21	6.3±0.7	81.2±0.17
0.1 ml. 5% beeswax in peanut oil	20†	9.0±0.8	82.8±0.11	7.2±0.8	85.2±0.14	10.6±1.1	83.6±0.60	8.5±0.7	82.6±0.5	5.2±1.4	81.5±0.31

Controls—Posted at 0–48 hours after injection of 0.2 ml. saline, PVP or 0.1 ml. beeswax-oil.

(For additional controls, see Table 1)

No. rats	Glycogen (glucose equiv.) μmoles./gm.	Water %
13	6.9±0.5	81.5±0.14

* All rats ovariectomized 13–15 days before use. All rats primed with 5 μg. ECP before these injections.
† 3–5 rats sacrificed at each time interval post injection.

TABLE 3. COMPARISON OF EFFECTS OF RELAXIN STANDARD AND LOW POTENT CONTROL EXTRACT W1164-A, LOT 36 ON COMPOSITION OF THE RAT UTERUS 24 HOURS AFTER A SINGLE INJECTION

Preparation	Dose (in 0.2 ml. 1% (L-390)		No. rats	Uterine weight, mg.	Uterine glycogen (glucose equiv.) μmoles/gm.	Uterine water %
	mg.	gpu.				
Lot 36*	1.5	15	13	227.2 ± 8.3	10.9 ± 0.7	83.0 ± 0.16
Relaxin Std.†	0.1	15	10	232.9 ± 10.3	11.5 ± 0.7	83.4 ± 0.12
Lot 36	0.1	1	13	206.0 ± 5.8	8.4 ± 0.5	82.4 ± 0.14
Relaxin Std.	0.0067	1	7	186.4 ± 10.5	9.3 ± 1.0	82.2 ± 0.21
Controls	0	0	12	162.4 ± 6.9	6.5 ± 0.6	81.4 ± 0.12

* Each mg. of Lot 36 is equivalent in potency to 0.07 mg. relaxin reference standard, or by old criteria has an activity of 10 guinea pig units/mg.

† Relaxin reference standard W1164-A Lot 8, assaying 150 guinea pig units/mg. by old criteria.

glycogen and water occurring during the estrus cycle of the rat. In chronic experiments to be reported elsewhere, there is little doubt that estrogen, even in high dosage, is incapable of producing the quantitative effects of relaxin on uterine and cervical composition. Nor can a combination of estrogen and progesterone produce such effects in rats or mice. Yet, relaxin, like progesterone, requires a priming treatment with estrogen to exert its most dramatic effects on the uterus.

In intact, estrogen primed mice, uterine glycogen deposition has been induced by dosages of relaxin which do not influence uterine weight nor increase the length of the interpubic ligament.

The glycogenetic activity of relaxin is best appreciated by comparison with values obtained by Walaas (8) after injection of a large dose (50 μg.) of estradiol benzoate in rats. 100 μg. of relaxin reference standard (which extract probably contains *a relatively small amount of the active principle*) produced as great an increase in glycogen in 12 hours as did the estrogen in 24–48 hours. Similarly, the increased uterine hydration observed by Zarrow (6) and ourselves when relaxin was administered to intact or primed castrate rats, occurred as rapidly and was as dramatic as that observed following estrogen treatment of castrates (11, 13). Continued estrogenization does not maintain high water levels (10), whereas repeated relaxin injections have been found to maintain water levels above 84%.

Finally, following estrogen treatment, uterine dry weight and nitrogen are reported to reach a maximum value about 30 hours post-injection (11, 13), whereas relaxin induced immediate nitrogen deposition which reached a peak in 12 hours. Uterine dry weight was highest between 6 and 12 hours after relaxin administration.

These observations, as well as data to be reported elsewhere, indicate that relaxin may play an important regulatory role in normal uterine metabolism.

SUMMARY

Twelve hours after a single subcutaneous injection of relaxin in a repository vehicle, there was observed a two-fold increase in the glycogen concentration of uteri of estrogen primed spayed rats.

This was preceded by a rapid and substantial increase in uterine weight due to an increase in water content, dry weight, and total nitrogen.

Acknowledgments

Gratitude is expressed to Mr. Lloyd Haynes, Miss Rhoda Nussbaum and Mrs. Frances MacKenzie for technical assistance, and to Mrs. Gladys Willever for animal care.

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THE EFFECT OF DESOXYCORTICOSTERONE ACETATE AND OF SODIUM ON THE JUXTAGLOMERULAR APPARATUS[1]

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THERE is considerable indirect evidence supporting Goormaghtigh's (1) hypothesis that the granular cells of the juxtaglomerular apparatus (JGA) constitute one phase of a secretory cycle which leads to the production of a renal vasomotor substance or a precursor thereof; yet the role of these cells, in the absence of more convincing evidence, must be considered as unknown. The morphological changes exhibited by these specialized elements, which are found at the vascular pole of the renal glomerulus in all mammals studied thus far (2), suggest a high degree of functional activity. The factors which modify or regulate these changes are of great interest because they may offer additional clues as to the function of the JGA.

Goormaghtigh (3) suggested that a lowered renal blood pressure or reduced oxygen tension of the renal blood are factors which cause an increase in the JGA granular cells and presumably an increase in secretory activity. Bohle (4) attributed the increase in JGA cells to a lowered systemic or intrarenal pressure, or to a reduced renal blood flow. Dunihue (5) found that adrenalectomy caused a tremendous increase in granular cells, which developed prior to a drop in the systemic pressure. He also reported that anoxic anoxia failed to increase these cells; while histiotoxic anoxia caused a moderate increase (6). This led him to suggest that the granular cells are increased in response to a disturbance in cellular oxidative mechanisms. Later, Dunihue, (7) and then Dunihue and Robertson (8) reported that mineralo-corticoids, as represented by desoxycorticosterone acetate (DCA), seem to regulate the granular cell phase of the JGA; since these cells were increased in cats with a DCA deficiency and decreased in those with an elevation of the DCA level. However, Schloss (9) reported no change in the JGA following adrenalectomy, and Doughtery (10) found the apparatus hypertrophied after injections of DCA. More recently, Hartroft and Hartroft (11) suggested that the influence of the adrenal on the JGA is secondary to a disturbance in salt metabolism. In a later paper

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(12) they reported parallel responses in the JGA and the adrenal zona glomerulosa to alterations of salt intake, and suggested that salt metabolism is the primary mediator of these changes.

The work here presented is a final report on the effect of various combinations of sodium intake and the level of mineralocorticoids, as represented by DCA, on the feline JGA.[2] It clearly shows a primary role for mineralo-corticoids in the regulation of the granular cell phase of the JGA in this animal. It also reveals that under certain conditions the action of one mineralo-corticoid, DCA, is facilitated by sodium.

MATERIAL AND METHODS

Cats were subjected to 8 of the 9 possible combinations of low, high and normal sodium intake with low, high and normal mineralo-corticoid levels. The majority of cats, secured from a commercial source, were males with an estimated age range of from 1 to 5 years. A few were females, but these are not reported separately because the data for them did not differ significantly from that for the males. The following groups of animals were studied:

I. Normal Mineralo-corticoid and normal sodium. This group was composed of 28 normal animals fed a regular stock diet of canned dog food, canned pilchards or fresh beef heart.

II. "Normal" Mineralo-corticoid and low sodium. The 18 cats in this group were fed Lonalac[3] suspended in distilled water. Dextrose, cod-liver oil or cream and dialyzed beef heart powder were added to the Lonalac in amounts sufficient to provide between 60 and 90 calories per kilogram per day. Vitamin supplement was added in the form of a concentrated pan-vitamin preparation. Eight of the cats in this group were subjected to peritoneal lavage or mercury diuresis (Mercuophylline, U.S.P.).

III. "Normal" Mineralo-corticoid and high sodium. The 4 normal cats in this group received in addition to the normal stock diet 2 gm. of sodium chloride daily per os for a period of 22 days.

IV. Low Mineralo-corticoid and normal sodium. The 10 bilaterally adrenalectomized cats in this group were supported for a period of 15 to 27 days by subcutaneous injections of 4 to 5 mg. of cortisone acetate[4] administered twice daily. Six of these animals were fed the normal stock diet and four were fed Lonalac diet to which sodium had been added in an amount which provided a normal sodium intake.

V. Low Mineralo-corticoid and low sodium. This group was composed of 13 adrenalectomized animals which, with the exception noted below, received no supportive or replacement therapy. They were fed the regular stock diet but are considered in the low sodium group because of the increased renal loss of sodium and the decreased dietary intake of sodium associated with anorexia. Two cats in this group were supported with cortisone and force-fed the Lonalac low sodium diet for from 9 to 15 days, thus providing a controlled caloric intake with a low mineralo-corticoid and low sodium. They did not differ from the other animals in this group.

VI. Low Mineralo-corticoid high sodium. The 5 adrenalectomized animals in this group were supported by subcutaneous injections of 4 to 5 mg. of cortisone acetate administered twice daily. These cats were fed the regular stock diet for a period of 15 days.

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[2] Reported as preliminary observations in references 7 and 8.

[3] A powdered food similar to whole milk in nutritional values but almost free of sodium. Mead Johnson and Company.

[4] Supplied by Merck and Company, Incorporated.

In addition each cat was given sodium chloride, intravenously and per os, so that the total sodium intake was in excess of the previous day's excretion.

VII. High Mineralo-corticoid and normal sodium. The 10 male cats in this group were castrated and unilaterally nephrectomized, which according to Selye (13) increases their sensitivity to DCA. They were fed the stock diet. Five received daily subcutaneous injections of 10 mg. of DCA[5] in sesame oil for 22 to 30 days, and the other 5 received the overdose of DCA in the form of implanted wafers for periods ranging from 35 to 54 days. The average weight of the wafers was 450 mg.

VIII. High Mineralo-corticoid and low sodium. This group of thirteen cats was force-fed a Lonalac-dextrose diet in an amount which provided 60 to 90 cal./kg./day. They received daily subcutaneous injections of 10 mg. of DCA in sesame oil for from 15 to 30 days. Four of this group were normal cats; 5 were castrated and nephrectomized and 4 were bilaterally adrenalectomized.

VIII. A. High Mineralo-corticoid and low sodium. The 4 adrenalectomized animals in this group were permitted to show signs of adrenal insufficiency (anorexia and weight loss) prior to beginning daily subcutaneous injections of 10 mg. of DCA and force-feeding the low sodium-Lonalac diet as in Group VIII.

The 9th possible combination of sodium and mineralo-corticoid, namely: high levels of both, was not studied since the results in group VII and VIII reveal that it could not shed further light on the relative affects of sodium and DCA.

All adrenalectomies were performed in two stages under aseptic conditions using sodium pentobarbital as the basic anesthetic, supplemented with ether when necessary. Before being placed on an experimental regime, all adrenalectomized animals were supported with approximately 1 mg./DCA/day for a period of two weeks.

Blood for electrolyte determinations was taken directly from the carotid artery or, in a few instances, directly from the heart. The heart was removed quickly and immediately prepared for electrolyte and water analysis which were made according to the methods described by Robertson (14). Electrolyte determinations were not done on all animals.

The kidney's were fixed in Helly's fluid, cleared in cedarwood oil imbedded in paraffin, sectioned at 4μ and stained with Masson's trichrome or Bowie's stain.

A granular cell index (GCI) was established for each kidney by the following method: All of the glomeruli in one or more sections of a kidney were counted and during this enumeration each occurrence of granular cells, whether singly or in groups, was recorded. The number of discrete occurrences of granular cells in each kidney was divided by the total number of glomeruli to give the granular cell index. This index, then, is a measure of the number of times one may expect to encounter granular cells, singly or in groups during the course of counting one hundred glomeruli. This index is easily reproducible by the same and by different observers, provided the entire thickness of the cortex is systematically surveyed. The number of granular cells at each encounter was also recorded as 1 to 2, 3 to 4, and more than 4. This data, however, is not presented, since it was found to vary in the same direction as the granular cell index.

RESULTS

In cats deprived of mineralo-corticoids, the granular cell index is higher than in normal animals (Figs. 1 and 2). This elevation is the result of metaplasia of arteriolar smooth muscle or afibrillar cells, but in addition there is hyperplasia of existing granular cells (Fig. 4) which may produce large masses of granular cells in the JGA (Fig. 5). On the other hand

[5] Generously supplied by the Schering Corporation.

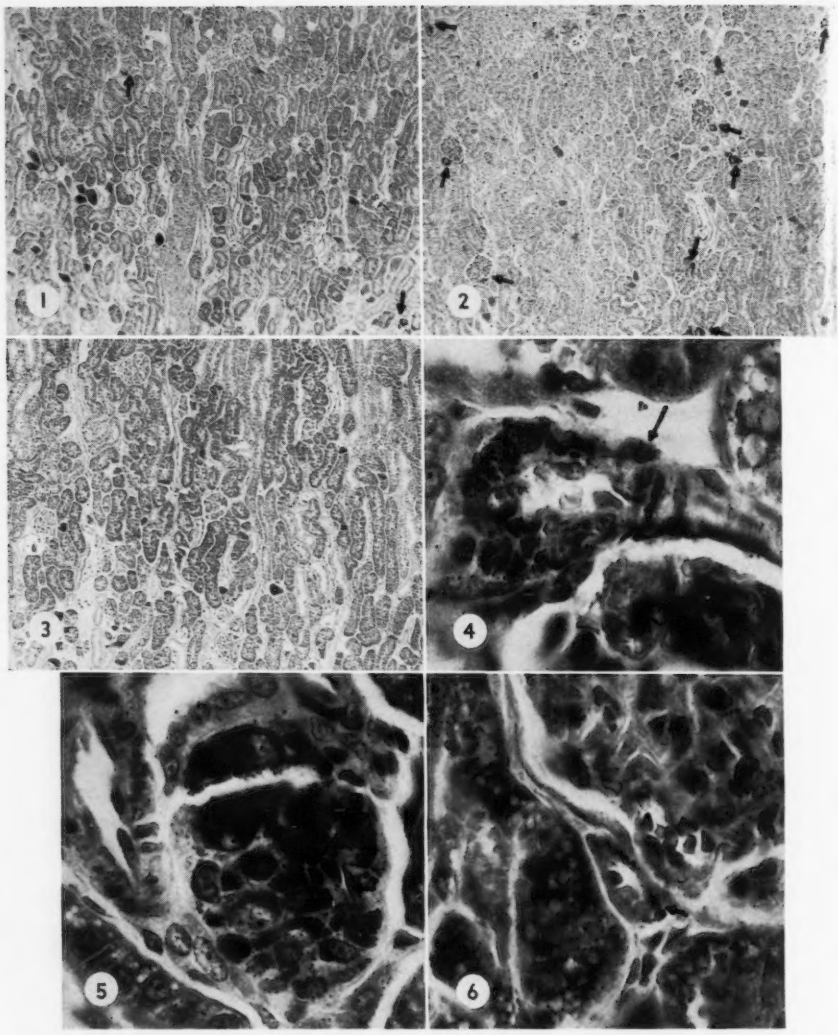

FIG. 1. Incidence of granular cells in a normal cat kidney. Granular cells are indicated by arrows. Bowie's stain. ×40.

FIG. 2. Increased incidence of granular cells in the JGA of a cat 17 days post-adrenalectomy. Normal diet, no support during terminal two weeks. The dark clusters indicated by arrows are composed of granular cells. Bowie's stain. ×40.

FIG. 3. Decreased incidence of granular cells in the kidney of a cat subjected to DCA overdose for 30 days. There are no granular cells in this field. Bowie's stain. ×40.

FIG. 4. Mitosis of a granular cell in the afferent arteriole of a cat 10 days post-adrenalectomy. Normal diet, no support. Bowie's stain. ×720.

FIG. 5. Metaplastic and hyperplastic JGA in a cat 17 days post-adrenalectomy. Normal diet, no support during terminal two weeks. Arrow point to lumen of the afferent arteriole. Bowie's stain. ×720.

FIG. 6. Reduction in size and granularity of JGA cells in castrated, unilaterally nephrectomized cat subjected to DCA overdose for 30 days. Bowie's stain. ×720.

elevated levels of DCA reduce the index (Fig. 3) as well as the size and granularity of the individual cells (Fig. 6).

The mean granular cell indices and the serum and tissue sodium concentrations are presented in Table 1.

These data reveal that the number of granular cells in the JGA, as measured by the GCI, is inversely related to the DCA level and is unrelated to the sodium intake. The GCI is increased when the mineralocorticoid level is low (Groups IV, V, VI), regardless of the sodium intake, and it is decreased by a high DCA level when the sodium intake is normal (Group VII). On the other hand, low and high sodium intake are associated

TABLE 1. RELATION OF DCA AND OF SODIUM TO THE GRANULAR CELL INDEX OF THE JUXTAGLOMERULAR APPARATUS

Group and treatment	Sodium, M.Eq.			Granular cell index
	Analytical data		Derived data	
	Serum[1]	Myocardium[2]	Intracellular[3]	
I Normal DCA Normal Na	159 ±0.6 (24)	19.3±0.5 (24)	6.5 ±0.8 (24)	15 ±1.2 (28)
II Normal DCA Low Na	147* ±2.6 (10)	17.5* ±0.8 (10)	2.7* ±0.8 (10)	13 ±1.8 (18)
III Normal DCA High Na	162 ±0.8 (4)	18.8±1.0 (4)	8.6 ±0.6 (4)	9 ±1.8 (4)
IV Low DCA Normal Na	155* ±2.0 (6)	20.0±2.6 (6)	11.5* ±2.6 (6)	33* ±2.3 (10)
V Low DCA Low Na	140* ±1.7 (11)	18.9±0.2 (11)	1.8* ±0.9 (11)	39* ±3.1 (13)
VI Low DCA High Na	159* ±0.9 (3)	17.0±0.2 (3)	8.6* ±1.4 (3)	36* ±0.9 (5)
VII High DCA Normal Na	162* ±0.5 (10)	20.9±0.6 (10)	8.4 ±1.3 (10)	2.9* ±1.5 (10)
VIII High DCA Low Na	147* ±2.6 (10)	17.7±0.8 (10)	4.3* ±1.4 (10)	15.7 ±1.4 (13)
VIIIA Insufficiency High DCA Low Na	139* ±2.0 (4)	20.0±0.3 (3)	1.5* ±0.9 (3)	43* ±1.9 (4)

The data are expressed as the mean ±its standard deviation. The number of animals for which the mean is presented is given in parenthesis. This number is not always the same for means within a group, since electrolytes were not determined in all animals.

[1] M.eq./kg. serum water.

[2] M.eq./100 gm. dry, fat-free, blood-free heart muscle.

[3] M.eq./kg. fiber water.

* These values differ significantly ($P < .05$) from normal.

with both normal and elevated indices, and normal sodium intakes are coexistent with a low, a normal and an elevated GCI.

The data also show that the GCI is not correlated with the serum or tissue sodium concentrations. The index is normal in Group III and elevated in Group VI, but the sodium values in these groups do not differ significantly from the normal values of Group I. Further, with decreased serum and intracellular sodium, the index is elevated in Groups VIIIA and V but normal in Groups VIII and II.

The relation of the number of granular cells to the level of DCA is not completely independent of sodium, as may be observed by consideration of the data from Groups VII, VIII and VIIIA. A high DCA level strikingly reduced the GCI below normal, and in some cases even to zero, when associated with a normal sodium intake (Group VII), but did not do so when the sodium intake was low (Group VIII), nor was a high DCA level able to reduce an index previously elevated by adrenal insufficiency when the sodium intake was restricted (Group VIIIA). Thus, although primary in its effect, the positive action of DCA in reducing the GCI is possible only in the presence of adequate sodium. To this extent, then, sodium facilitates the action of DCA.

Our conclusion that the number of JGA granular cells is inversely related to the DCA level and that the positive action of DCA in reducing the number of granular cells requires adequate sodium is consistent with all of the data secured in cats and, with the exception discussed below, with the results reported for rats. On the other hand, the assumption of the Hartrofts that the GCI is inversely related to the sodium intake and directly with the activity of the Zona Glomerulosa—hence mineralocortiocids—and that the effect of sodium is primary can explain the GCI in only two groups of Table 1 on the basis of intake and intracellular sodium and of only four groups on the basis of serum sodium. We suggest, therefore, that our interpretation, since more data in both the cat and rat are accordant with it, more nearly reflects the events of GCI regulation.

The experimental condition in which the rat differed from the cat, and in which the data are not readily explained by the mechanism we have suggested, was that in which normal animals were fed a low sodium diet. In the rat the number of granular cells increased, whereas in the cat it remained normal. The possibility that our low sodium diet, because of other deficiencies, inhibited the development of granular cells or caused them to regress is precluded by the large number of granular cells which developed in adrenalectomized, cortisone supported cats fed the Lonalac diet supplemented with sodium chloride (Group IV).

In the absence of other apparent causes, it seems likely that this difference in the response of the rat and the cat to low sodium intake may be due to species difference, for adrenalectomized rats can be maintained by the simple procedure of adding sodium chloride to their drinking water,

while cats cannot. Also, it may be that the adrenals in these species respond differently to a low sodium stimulus. If this response in the rat, but not in the cat, were to result in a temporary reduction in release and production of mineralo-corticoid, then the granular cell index should be elevated in the rat and normal in the cat.

SUMMARY

The relative roles of a mineralo-corticoid, desoxycorticosterone acetate, and of sodium in regulating the number of granular cells in the juxtaglomerular apparatus were investigated in cats subjected to various combinations of low, normal and high DCA levels with low, normal and high sodium chloride intakes. The number of granular cells was found to be inversely related to the DCA level and unrelated to the sodium intake. Further, the positive action of high DCA levels in reducing the number of these cells was facilitated by sodium. It is concluded that mineralo-corticoids, as represented by DCA, have a primary role in regulating the granular cells of the juxtaglomerular apparatus.

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EFFECTS OF A BACTERIAL POLYSACCHARIDE (PIROMEN®[1]) ON THE PITUITARY-ADRENAL AXIS: ADRENAL ASCORBIC ACID, CHOLESTEROL AND HISTOLOGIC ALTERATIONS

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INTRODUCTION

IMPROVEMENT has been reported in a variety of disease conditions after the administration of pyrogenic substances (1). Piromen®, a bacterial polysaccharide complex of *Pseudomonas* origin, has been effectively used for the relief of allergic conditions (2–4), dermatologic upheavels (5, 6), opthalmologic dyscrasias (7), vascular (8) and central neuronal pathology (9). Kirkendall *et al.* (10), Conn and his associates (11), and Wexler and co-workers (12) have shown that Piromen stimulates the pituitary gland to release corticotropic hormone with attendant adrenal cortical sequelae.

A series of fundamental endocrine studies was undertaken in order to categorize, more fully, the adreno-cortical response to Piromen, both qualitatively and quantitatively. Adrenal ascorbic acid, cholesterol and histologic alterations served as the point of primary focus.

METHODS

Both intact and hypophysectomized male, Sprague-Dawley rats, weighing from 80 to 120 gm., were employed in these studies. Piromen, in a single intraperitoneal[3] injec-

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[1] Piromen® is a sterile, nonprotein and non-anaphylactogenic bacterial polysaccharide complex. It is prepared as a stable suspension in $\frac{1}{6}$ molar sodium R-lactate containing 1:10,000 parts of thiomerosal. Piromen is a product of Travenol Laboratories, Inc., a subsidiary of Baxter Laboratories, Inc., Morton Grove, Illinois.

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[3] Although clinical and experimental evidence indicates that Piromen is most effective when given intravenously, we gave this preparation intraperitoneally in order to avoid all extraneous and undue stress which might follow tail-vein injection. For the same reason, anesthesia was not induced and the rats were sacrificed by decapitation.

tion, was administered at several dosage levels[4] based on 100 grams body weight. Animals in control groups received $\frac{1}{6}$ *M* sodium D-lactate only. After hypophysectomy, animals were allowed a 24-hour period for recuperation before the test substances were administered. The animals were sacrificed by decapitation; the adrenal glands were removed as quickly as possible, trimmed free from fat, carefully weighed and homogenized in 2.5% metaphosphoric acid. The ascorbic acid content of the individual glands was determined according to the method of Mindlin and Butler (13). Adrenal cholesterol determinations were conducted by the procedure described by Schoenheimer and Sperry (14). Adrenals removed for histologic study were fixed in 10% buffered neutral formalin (Lillie) and the frozen sections cut to a thickness of 8 micra; these were stained for lipid content definition with Sudan orange (hematoxylin counterstain) and with Sudan black B (Mayer's carmalum counterstain).

RESULTS

Time Response to Piromen

A. *Ascorbic Acid.* Single injections, 10 μg. each, of Piromen were given to animals in several groups; these were sacrificed by group at hourly intervals to 6 hours and at 12 and 24 hours after injection. Guided by certain empirical earlier explorations we selected changes in adrenal ascorbic acid and cholesterol levels, along with histologic alterations in the gland, for our indices of ACTH release (15).

The response to Piromen, as shown by ascorbic acid depletion, was immediate. Within one hour the level of ascorbic acid was lowered in a manner and to a degree reminiscent of ACTH. However, unlike the response to ACTH, active depletion continued, to reach a maximum in 3 to 4 hours. This depletion was followed by a very rapid phase of ascorbic acid repletion so the normal glandular content was restored within 24 hours (Table 1).

B. *Cholesterol.* The adrenal cholesterol depletion-repletion phases were almost identical with those changes described for ascorbic acid although the depletion in cholesterol was less precipitous. Again, the time of maximum depletion occurred approximately 4 hours after the injection (Table 1).

Dose Response to Piromen

Once we had established the time of maximal ascorbic acid depletion between the third and fourth hour after the injection of Piromen, we gave single, graduated doses ranging from 1 to 50 μg. to a second group of animals. Between 1 and 10 μg. the graduated doses were given in increments of 1 μg.; above 10 μg. and up to 50 μg. the Piromen was given in increments of 5 μg. These were sacrificed four hours later. A graphic plotting of ascorbic acid depletion levels after Piromen describes a straight-line progression response after dosages between 1 and 10 μg.; above 10 μg. the depletion of

[4] In the human subject, doses as low as 4 to 5 gamma may induce marked increases in body temperature when given intravenously. In the cat and rabbit one gamma/kg. given intravenously will also stimulate a pyrogenic response.

TABLE 1. ADRENAL ASCORBIC ACID AND CHOLESTEROL CHANGES IN THE RAT FOLLOWING A SINGLE INTRAPERIONTEAL INJECTION OF PIROMEN. (10 μG./100 GM. BODY WT.)*

Hours after treatment	Mg. ascorbic acid per 100 gm. adrenal wt. with std. error		P value	Gm. cholesterol per 100 gm. adrenal wt. with std. error		P value
0	436	8.9	—	3.37	0.14	—
1	295	23.7	0.0001	3.11	0.17	0.2380†
2	—	—	—	2.60	0.17	0.0005
3	254	10.3	0.0001	1.93	0.18	0.0001
4	274	17.3	0.0001	1.65	0.10	0.0001
5	292	11.6	0.0001	1.71	0.14	0.0001
6	325	26.0	0.0002	2.40	0.08	0.0001
12	409	4.1	0.0078	2.60	0.14	0.0001
24	435	5.9	0.9283	3.04	0.11	0.0643†

* 8 animals per group.
† Not significantly different from controls.

ascorbic acid was increased but the degree of response, when plotted on a curvilinear basis, was of lesser slope. Figure 1 depicts the effect of 5, 10, 20, 30, 40 and 50 μg. of Piromen.

Effects of Hypophysectomy

In order to critically check the hypothesis that Piromen stimulates the adrenal cortex through the endogenous release of ACTH, groups of intact

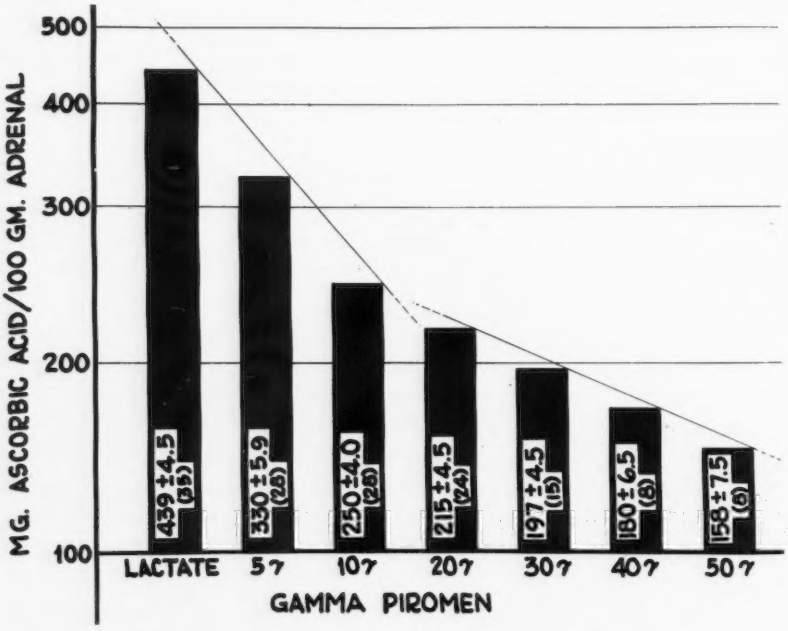

FIG. 1. Effect of single, graduated doses of Piromen (5 to 50 gammas), given intraperitoneally, on the adrenal ascorbic acid level of the intact, male, Sprague-Dawley rat. Animals were sacrificed four hours after injection.

and of hypophysectomized rats were given either injections of Piromen at a dosage of 10 μg. or the lactate placebo. When sacrificed 4 hours later the intact animals given the lactate placebo alone had not responded while there was a very active, significant depression of adrenal ascorbic acid in Piromen-treated animals. Four hours after a single injection neither the placebo nor Piromen brought depletion of the ascorbic acid content in the adrenal of the hypophysectomized rat (Fig. 2). The level of ascorbic acid in the adrenal is said to be frozen, i.e., specifically depleted by ACTH, 24 hours after the "stress" of hypophysectomy (16).

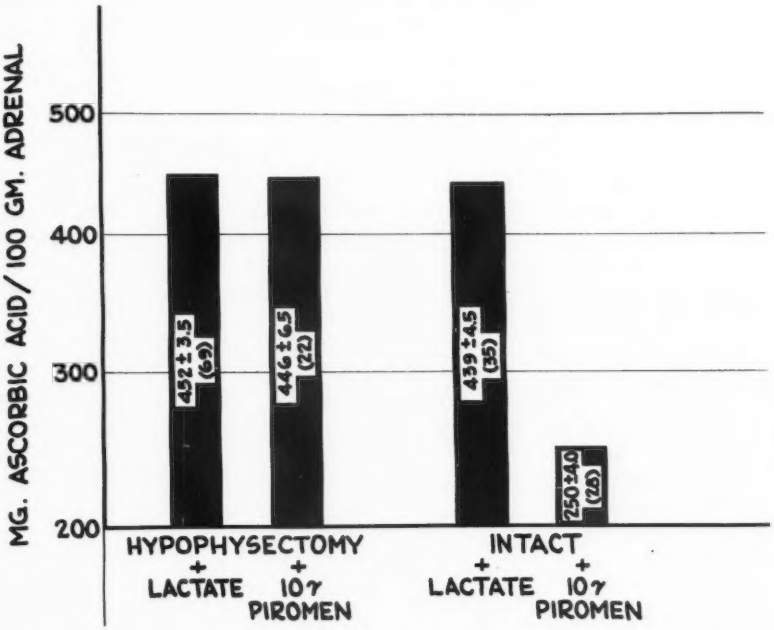

FIG. 2. Effect of hypophysectomy on the adrenal ascorbic acid depleting effect of Piromen. Note the complete absence of response to Piromen in the hypophysectomized animals.

Correlation of Adrenal Histologic Alterations and Quantitative Chemical Changes with Dose

As a separate facet of this study an attempt was made to determine whether or not correlation existed between histologic change and quantitative chemical alterations and if either was related to the size of the Piromen dose. Animals in several groups were given single injections of Piromen in multiples ranging from 5 to 50 μg.; at each graded dosage-level, the adrenals were removed at hourly intervals from 1 to 6 hours, and 12 and 24 hours after injection. The left adrenal was used for determination of ascorbic acid, the right for histologic examination.

In confirmation of our findings in previous studies, each dose-increment gave a proportional increase in response as measured by the maximal depletion of ascorbic acid at the fourth hour. While Piromen, in amounts below 25 μg., brought marked depletion of both ascorbic acid and cholesterol, alterations in the zonal lipid distribution pattern and in other cytologic elements were not apparent. Such changes are usually found after ACTH administration with ascorbic acid depletion (17, 18). Histologic changes were found when the dosage level reached 25 μg. (Fig. 3).

Preparations which we made from the adrenals of control animals and from those sacrificed 1 hour after Piromen showed no alteration histologically; when sacrificed 3 hours after treatment there was intense depletion of sudanophilic material. This depletion extended from the juxta-medullary border of the zona reticularis into the outer portions of the zona fasciculata. A well-defined, lipid-free, zone appeared between the zona glomerulosa and zona fasciculata (cf. Figs. 4–7). Such a lipid-free zone appears in the rat adrenal following an adequate dose of ACTH. This relatively "sudden" lipid depletion occurred simultaneously with the period of maximal depletion of ascorbic acid from the adrenal gland; furthermore, the capillaries travers-

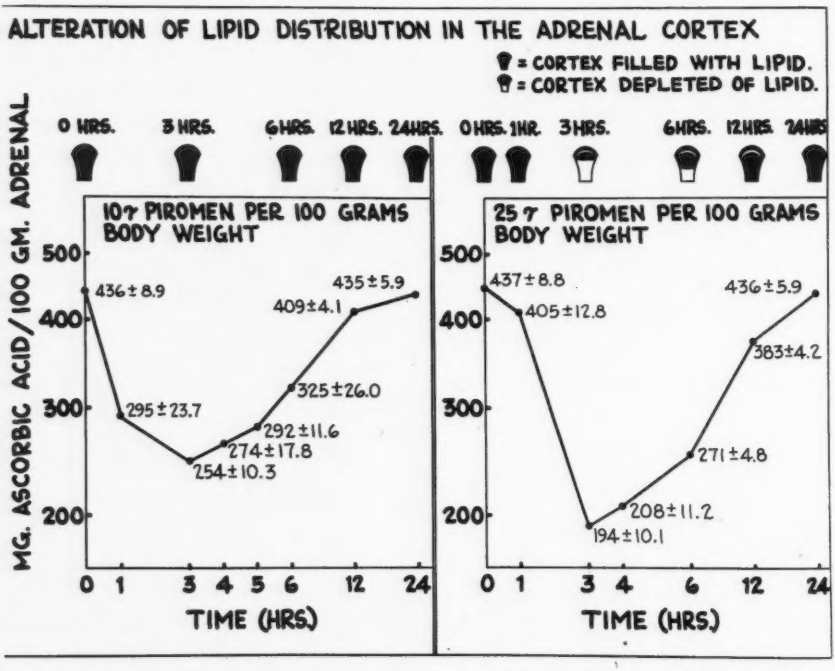

FIG. 3. Qualitative and quantitative changes in adrenal ascorbic acid levels and histology between two doses (i.p.) of Piromen, 10 and 25 μg. Note that at the 10 μg. level significant ascorbic acid depletion occurred without the usual concomitant changes in lipid distribution. Contrast this with animals given 25 μg.

ing the zona fasciculata and reticularis were conspicuously dilated, suggesting an active cortical discharge. There were no alterations in the distribution of the physical characteristics of sudanophilic material of the zona glomerulosa (Figs. 4–7).

Six hours after the initial injection and coincident with the beginning of the period of significant repletion of chemically demonstrable ascorbic acid, the outer and inner fasciculate zones began to refill, in a centripetal direction, with lipid. Within 12 hours the entire lipid complement was restored. After 24 hours the only remaining evidence of activity in an otherwise normal pattern, was the persistence of a lipid-free zone, then barely discernible. Ascorbic acid levels had, by this time, been restored to the normal range.

DISCUSSION

The difference in the degree and time of depletion of the adrenocortical stores after the release of endogenous ACTH by Piromen was compared with those found after other stimuli were given and was our first indication that the response might be qualitatively different. Maximum depletion of ascorbic acid after a single exposure to "stress" or a single injection of ACTH usually is between 30 and 50% (19); after Piromen it may fall to 60 or 70%. Similarly, cholesterol is depleted by 40 to 50% after ACTH while the decline lies between 60 and 80% after Piromen. Characteristic of the release of ACTH induced by Piromen is the appearance of the period of maximal depletion of cholesterol after 4 hours.[5] In our own work, as in that of other investigators, ACTH has shown its most pronounced effects one hour after administration. It is interesting to observe that, while persistence of the phase of depletion was protracted after Piromen treatment, there was no delay in the time of repletion of ascorbic acid or cholesterol. This portion of the "curve" is similar to that seen after the response to ACTH despite the fact that maximum depletion is reached earlier.

These experimental observations show good correlation with the results observed by Conn in humans (11). In comparing the adrenal metabolic response to ACTH and Piromen, Conn observed that the response to Piromen is slower in development and of longer duration than the metabolic consequences which follow ACTH.

The fact that increased Piromen dose increments produced quantitatively greater ascorbic acid depletion for the respective levels suggests that release of endogenous ACTH by Piromen may be caused by other than a "non-specific" stress stimulus as suggested by Conn (11). The absence of thymus involution in the presence of maximal ascorbic acid depletion lends support to this contention.

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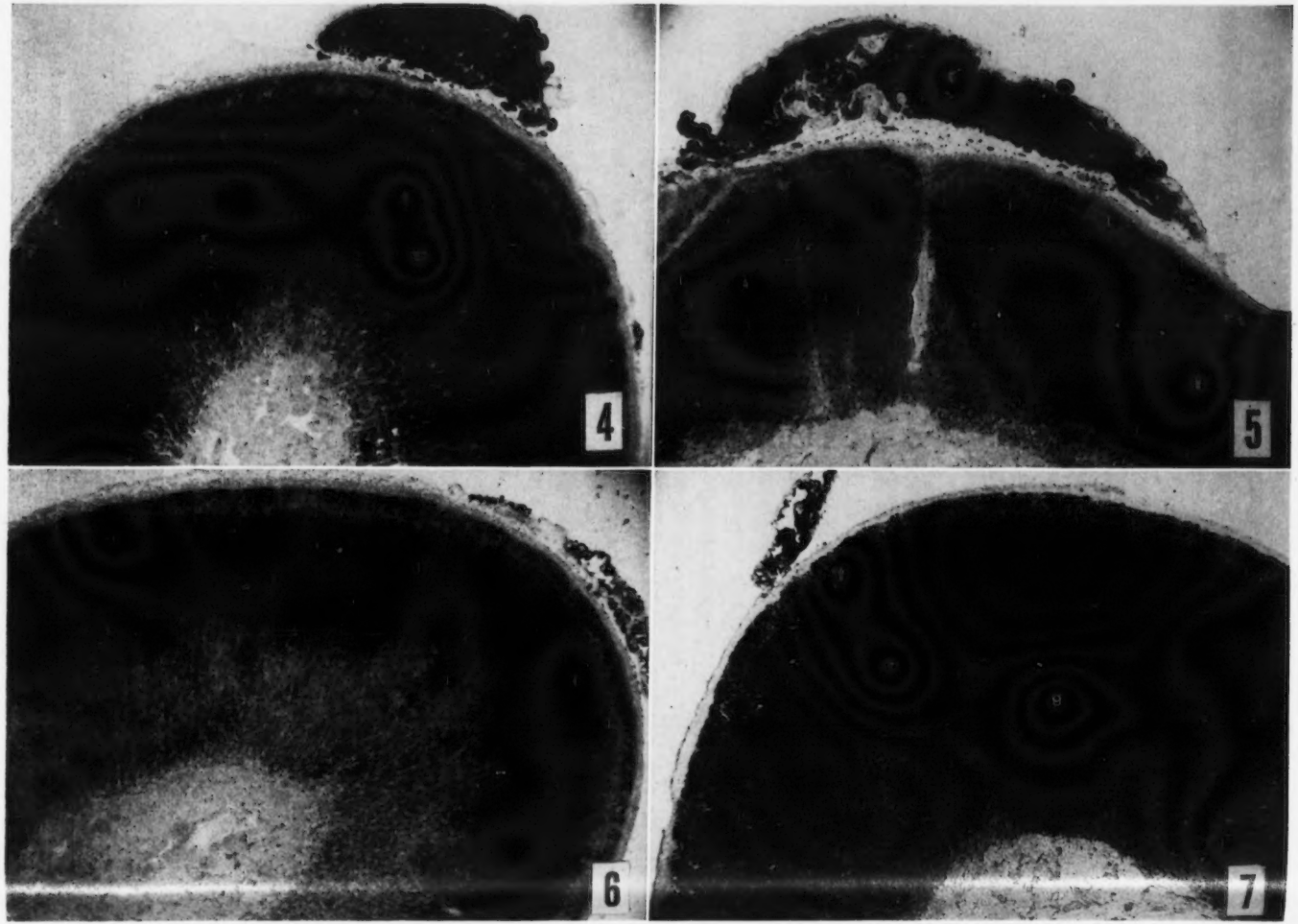
[5] The maximum rise in body temperature in the human subject after the injection of Piromen usually occurs in 4 hours; this is also true in the case of the monkey, dog, cat and rabbit.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The lack of response to Piromen in the hypophysectomized rat demonstrates clearly that Piromen increases adrenal activity through its ability to stimulate the pituitary gland in this species (see below). Piromen also simulates ACTH in its effect on the circulating granulocytic and agranulocytic blood elements (10). Piromen, but not ACTH, has been reported (20) to produce significant leukopenia and leukocytosis in the adrenalectomized dog. Others have reported changes in blood cellular components in adrenalectomized animals and in Addisonian patients (21–23). Such reports tend to weaken the concept that these changes in the blood cell are under absolute control by the pituitary-adrenal system. It is generally agreed, however, that results based on observation of the adrenalectomized animal can be very misleading and should be considered with some reservation. Piromen caused no changes in the circulating blood elements of the hypophysectomized rat (12). However, in the hypophysectomized cat, leukopoenia with fever followed by slight leukocytosis has been reported (24).

The changes in histologically demonstrable cortical lipids were, likewise, not identical with those which follow the administration of ACTH. It is only under unusual circumstances that adrenal activity, made evident by changes in the ascorbic acid and cholesterol contents, is not accompanied by histologic alterations (25–27), yet Piromen, in amounts below 25 μg., produces alterations in both ascorbic acid and cholesterol contents of the adrenal exceeding those found after ACTH without causing these cellular changes. At higher dosage levels (above 25 μg.) the changes were also unusual. Paradoxically, these changes were akin to those which follow the stimulatory action of ACTH—depletion of lipid from the zona reticularis and zona fasciculata and opening of a lipid-free zone—but were not evident until three hours after the injection of Piromen.

SUMMARY

The effects of a bacterial polysaccharide complex, Piromen, on the pituitary-adrenal system of the intact and hypophysectomized rat are described.

FIG. 4. Adrenal cortex of an intact, male, Sprague-Dawley rat. No treatment. Cortex contains a full complement of lipid. Sudan orange (hematoxylin). ×60.

FIG. 5. Adrenal cortex of an intact, male, Sprague-Dawley rat, 1 hour after a single intraperitoneal injection of 25 μg of Piromen/100 gm. b. w. No evidence of lipid depletion. Sudan orange (hematoxylin). ×60.

FIG. 6. Adrenal cortex 3 hours after 25 μg. of Piromen. Note clearly defined lipid-free zone and intense depletion of lipid from the zonae reticularis, inner fasciculata, and extending into the outer fasciculata. Sudan orange (hematoxylin). ×60.

FIG. 7. Adrenal cortex 24 hours after 25 μg of Piromen. The cortex is hypertrophied, the lipid-free zone barely discernible, and the lipid complement restored to normal. Sudan orange (hematoxylin). ×60.

In the intact rat given a single, intraperitoneal injection of Piromen, the changes in adrenal ascorbic acid and cholesterol indicate that this bacterial product stimulates the release of endogenous ACTH. Certain qualitative differences between the ascorbic acid and cholesterol responses to Piromen and ACTH are discussed.

In hypophysectomized rats ascorbic acid depletion does not occur following the administration of Piromen. This indicates that the pituitary gland plays an essential role in the endocrinal responses to Piromen.

A comparison of the histologic changes in the adrenal glands with the chemical differences reveals some interesting variations in the adrenal response to Piromen and ACTH.

It is postulated that Piromen induces increased adrenal cortical activity by virtue of a direct stimulatory effect on the pituitary gland rather than as a non-specific stressing agent.

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NOTES AND COMMENTS

DURATION OF ALLOXAN DIABETES IN THE HAMSTER[1]

The reports of several investigators have indicated a high degree of variability in the duration of alloxan diabetes. Although Phatak, Lal and Everett (1) implied that hyperglycemia in hamsters persists almost indefinitely, it soon became apparent that a high percentage of our animals recovered early.

Since our particular interest lay in the use of diabetic animals during a period of chronic hyperglycemia, it became necessary that long term observations be made.

METHODS

Two major groups of hamsters were used. The first was injected intravenously with the diabetogenic dose of Eastman alloxan proposed by Nace, House and Tassoni (2). These were left undisturbed for 8 weeks. At that time, and at weekly intervals over a period of several months thereafter, blood was analyzed for glucose by the method of Durham, *et al.* (3). The second group received alloxan in the same manner and at the same rate. However, these were tested 24 hours after injection,[2] again after 4 days and then at weekly intervals for the next 2½ months. Subsequently, analyses were made every 2 to 4 weeks for 5 months.

OBSERVATIONS

The type of response to alloxan was classified as follows: negative, in which no significant change in blood sugar is ever seen; fatal, in which death occurs within 5 days; diabetic, in which blood sugar is significantly elevated. The latter category was further subdivided into: transient, in which blood sugar returns to permanently normal levels within 4 weeks; persistent, in which hyperglycemia is maintained for at least 8 weeks; intermittent, in which blood sugar returns to normal one or more times only to rise again to diabetic levels.

TABLE 1

Group 1. Animals, nearly equally divided according to sex, were injected but not tested for 8 weeks. At the left is given the numbers of animals used. In the next column is shown the percentage of animals still alive at eight weeks. On the right, the percentage of survivors still diabetic at 8 weeks is given.

Animals used		% surviving 8 wks.	% surviv. diabet. 8 wks.
Females	35	65.7	39.1
Males	30	50.0	33.3
Total	65	58.5	36.8

The results of our finding in the first group are summarized in Table 1. It should be recorded that 2 in the persistent class recovered between 6 and 10 weeks while one remained diabetic for more than 22 weeks.

Statistics based upon earlier experiments indicated that at least 40 animals should

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TABLE 2

Group 2. Hamsters injected but tested at frequent intervals beginning 24 hours after treatment. The total number of animals used is shown at the left. Progressing toward the right, the percentage values for the following categories are given. neg.—no blood sugar changes; fat.—animals dead because of alloxan; diabetic—animals whose blood sugar rose above 130 mg. %. The diabetics were classified thus: trans.—animals recovering within 4 weeks; persist.—animals whose blood sugar remained elevated 8 or more weeks; intermit.—hamsters showing two or more periods of hyperglycemia alternating with normal periods over an interval of 8 weeks.

No. animals	Reaction				
	Neg.	Fat.	Diabetic 58.1%		
			Trans.	Persist.	Intermit.
117	21.4%	20.5%	44.1%	39.7%	16.2%

have responded to alloxan and survived. Since the present report showed only 14 to be diabetic at 8 weeks, it was obvious that a large number must have recovered. Thus, it was necessary to explore those weeks immediately following the alloxan injection. The results are summarized in Table 2.

Among the animals classified as transient diabetic, 70% recovered within 14 days.

In the persistent class, all who were diabetic at 4 weeks were still diabetic at 8 weeks. Only 6 animals were actually followed over the entire 7 month period. Of these, 4 recovered by the sixteenth week. Two others were still hyperglycemic after 30 weeks.

In the intermittent class, no consistent pattern was observed. However, about 70% maintained an elevated blood sugar for 3 to 4 weeks before the initial decline to normal. However, all of these, regardless of the time of the primary fall, were hyperglycemic again at 5 and 6 weeks. Two members of this group remained in an elevated state for 6 to 7 weeks, returned to normal but rose again 2–3 weeks later.

The blood sugar curves for the 3 diabetic types are interesting. Among the transients, a high of 200 mg.% was attained on the fourth day with the return to normal occurring between 10 and 12 days. The severely diabetics reached levels of 290 mg.% or higher, usually between 8 and 12 days. This was maintained for 40 or more days with a trend toward normal between 60 and 80 days. The intermittently diabetic showed a rise in blood sugar to between 200 and 250 mg.% within 4 days which persisted for about 24 days. Generally glucose values were rising again in 30 to 35 days. The second peak is not as high as the first but it may be maintained 30 days or more. A few animals showed a third rise,usually of shorter duration and lower amplitude.

In order to determine the effects of certain other factors, 67 animals were grouped according to age and sex. The results appear in Tables 3 and 4.

TABLE 3

The animals in this group were 8 to 9 weeks of age at the time of injection. They were segregated according to sex but were treated and classified as indicated under table 2.

No. animals	Reaction				
	Neg.	Fat.	Diabetic 54.3%		
			Trans.	Persist.	Intermit.
Females 21	14.3	33.3	81.8	18.2	0.0
Males 14	21.4	21.4	12.5	25.0	62.5
Total 35	17.1	28.6	52.6	21.1	26.3

TABLE 4.

The animals in this group were 6 to 8 months of age at the time of injection. They were segregated according to sex but were treated and classified as indicated under table 2.

No. animals	Reaction				
	Neg.	Fat.	Diabetic 68.7%		
			Trans.	Persist.	Intermit.
Females 13	23.1	0.0	50.0	20.0	30.0
Males 19	5.3	31.6	66.6	16.7	16.7
Total 32	12.5	18.8	59.1	18.2	22.7

DISCUSSION

The classification of response used herein is a modification of two systems, one proposed by Beach, *et al.* (4), the other by Wrenshall and coworkers (5).

Our observations merely serve to emphasize the differences in the persistence of the diabetic syndrome, particularly among rodents.

The age and sex differences were not significant in many particulars and warrant no further comment here.

In our earlier work, the intermittent classification was overlooked. Since the majority of these reached normal limits for the first time during the same period in which the transient diabetic may recover, it is probable that most of the intermittents were placed in the transient category.

SUMMARY

In a group of 65 hamsters injected intravenously with a diabetogenic dose of alloxan, 36.8%, surviving for 8 weeks, were still diabetic. A second group, consisting of 117 animals, was similarly treated but was tested frequently over a period of several months. It was found that 58.1% remained diabetic for variable periods. Of these, 39.7% were persistent diabetics, 44.1% were transient diabetics while 16.2% were diabetics of the intermittent type. An attempt was made to demonstrate age and sex differences but the results were inconclusive.

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FURTHER STUDIES ON HORMONAL FACTORS AFFECTING THE NUMBER OF CIRCULATING EOSINOPHILS IN THE GUINEA PIG

Evidence is accumulating that hormones other than those from the adrenal cortex effect the number of circulating eosinophils in the normal animal (1, 2, 3, 4). A sex difference in eosinophil levels in normal adult guinea pigs has been shown due, at least in part, to an eosinopenic effect of male sex hormone (5). Two questions raised in the latter report are subject of this investigation: 1) Does an eosinophilic influence of estrogens have a role in maintaining the higher eosinophil levels characteristic of the adult female guinea pig? 2) Does testosterone induce the eosinopenia directly, or via the pituitary-adrenal axis?

METHODS

Alterations in the number of circulating polymorphonuclear leucocytes and eosinophils—determined by direct chamber counts (5, 6)—were followed in 12 female guinea pigs spayed when immature (29 to 47 days old), and in 10 of these hypophysectomized 7 months after spaying. The effects of estradiol (20 μg./day of Progynon[1] for 5 days) in the spayed and the spayed-hypophysectomized animals, and of testosterone (500 μg./100 gm. body weight of Oreton-F[1] daily for 7 days) in the spayed-hypophysectomized animals, were also studied. Both hormones were given in aqueous suspension by subcutaneous injection. Completeness of hypophysectomy was confirmed at autopsy by examination for sella fragments and determination of adrenal weights ($40 \pm .031$ mg./100 gm. body weight). The white blood cell levels of 100 normal female guinea pigs, from our colony and within the age-range of the experimental animals, were determined for comparison.

RESULTS AND DISCUSSION

The data are summarized in Tables 1 and 2. Eosinophils have been reported both as cells/mm.^{3} and cells/1000 polymorphonuclear leucocytes because alterations in the latter figure may reveal an eosinopenia which would otherwise be masked when a concomitant release of granulocytes is adding eosinophils to the circulating blood (7).

The present study offers additional evidence that gonadal hormones affect the normal levels of circulating eosinophils: 1) The mean number of these cells in 50 normal females which were more than 3 months old was significantly above (+44%) that in 50 normal females less than 3 months old. The breakdown into smaller age-ranges shows that this increase in eosinophils occurred quite abruptly after the 3rd month and was sustained virtually unchanged even in the group 155 to 350 days old. 2) Females spayed when less than 2 months old maintained the pre-pubertal level of eosinophils even 7 months after operation. 3) Estradiol administered to the spayed guinea pigs raised the eosinophils/mm.^{3} to almost three times the control level, and the eosinophils/1000 polymorphonuclear leucocytes to more than twice as high as in the controls.

Thus, the sex difference in the level of circulating eosinophils in the normal adult guinea pig (1, 2, 3) can be explained in terms of an eosinophilic effect of estrogen as well as the previously demonstrated (5) eosinopenic effect of androgen. It is interesting to note that Halberg, Hamerston and Bittner (4) have found in certain strains of mice that it is the male which has the higher eosinophil level, suggesting the possibility that there may be species differences in the action of gonadal hormones on these cells.

Some reports in the literature may be explicable in terms of this eosinophilic action of estrogen, although the fact that the number of polymorphonuclear leucocytes were not stated makes it difficult to distinguish between specific eosinophilias and increases in these cells as the result of a general release of granulocytes. Dworetsky, Code and Higgins (1) found that pregnancy abolished the eosinopenic response to ACTH in the guinea

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[1] Progynon and Oreton-F were kindly supplied by Dr. Milton Eisler, Schering Corporation, Bloomfield, N. J.

TABLE 1

Age (days)	Number of animals	Polymorphonuclear leucocytes cells/mm.^{3} ± S.E.	Eosinophils cells/mm.^{3} ± S.E.	Eosinophils/1000 polymorphonuclear leucocytes
17–90	50	1937 ± 110	109 ± 9	56.3
95–350	50	2854 ± 270*	157 ± 15*	55.0
17–30	14	1916 ± 262	101 ± 14	52.7
35–57	20	1667 ± 113	110 ± 13	65.9
65–90	16	2295 ± 215	113 ± 19	49.2
95–145	36	2688 ± 225	158 ± 18	58.7
115–350	14	3205 ± 508	154 ± 26	48.0

* Highly significant difference from group 17–90 days old: $P < .01$.

TABLE 2

Experimental procedure	No. of animals	Polymorphonuclear leucocytes cells. mm.^{3} ± S.E. (% change)	Eosinophils cells. mm.^{3} ± S.E. (% change)	Eosinophils/1000 polymorphonuclear leucocytes (% change)
Effect of Spaying				
Control: Before spaying (29–47 days old)	12	1732 ± 164	107 ± 17	61.8
Spayed:				
3 months	12	3471 ± 803* (+ 99.8%)	109 ± 13 (+1.8%)	31.4 (−49.2%)
4 months	12	4770 ± 616† (+175.4%)	105 ± 19 (−1.8%)	22.0 (−64.4%)
6 months	12	3580 ± 372† (+106.7%)	108 ± 17 (+0.9%)	30.1 (+51.3%)
Effect of Estradiol (Spayed)				
Control: Spayed 6 months	12	3580 ± 372	108 ± 17	30.1
Injected: (5 days)				
after 3rd inj.	12	5380 ± 409† (+ 50.3%)	213 ± 30† (+ 97.2%)	39.5 (+ 31.2%)
off 1 day	12	5750 ± 633† (+ 60.6%)	414 ± 66† (+283.3%)	70.4 (+133.8%)
off 5 days	12	4609 ± 399 (+ 28.7%)	148 ± 25 (+ 37.4%)	32.1 (+ 6.6%)
off 8 days	12	3638 ± 372 (+ 1.6%)	80 ± 17 (− 25.9%)	21.9 (− 27.2%)
off 19 days	12	3587 ± 338	105 ± 20 (− 2.8%)	29.3 (− 2.6%)
Effect of Hypophysectomy (Spayed)				
Control: Spayed 7 months	10	3760 ± 395	111 ± 24	29.6
Hypx.: 32 days	10	3050 ± 253 (− 18.8%)	232 ± 41† (+109.0%)	76.0 (+156.7%)
Effect of Estradiol (Spayed-Hypx)				
Control: Hypx 32 days	10	3050 ± 253	232 ± 41	76.0
Injected: (5 days)				
off 1 day	10	6340 ± 570† (+107.8%)	1008 ± 212† (+334.4%)	159.2 (+109.4%)
off 5 days	10	4165 ± 568 (+ 36.5%)	709 ± 121† (+205.6%)	170.2 (+123.9%)
off 8 days	10	2685 ± 201 (− 11.9%)	215 ± 27 (− 7.3%)	80.8 (+ 6.3%)
Effect of Testosterone (Spayed-Hypx)				
Control: Hypx 2 months	10	3265 ± 363	230 ± 49	70.4
Injected: (7 days)				
off 1 day	10	2645 ± 305 (− 18.9%)	16‡ (− 93.1%)	6.1 (−91.3%)
off 8 days	10	3540 ± 237 (+ 8.4%)	11‡ (− 95.2%)	3.1 (−95.6%)

* Significant difference from control: $P < .05$.
† Highly significant difference from control: $P < .01$.
‡ Too few cells for reliable determinations.

pig; and Louwrens and Smelik (8) reported that implants of estradiol prevented an eosinopenia one hour after painful stimuli in this species.

In the spayed immature female, although the eosinophils/mm.^{3} were unchanged after operation, the eosinophils/1000 polymorphonuclear leucocytes fell to half those characteristic of normal females. This decline in the *proportion* of eosinophils (in the face of a rise in polymorphonuclear leucocytes) was interpreted as an eosinopenia induced by adrenal cortical hormone no longer counterbalanced by the eosinophilic effect of estrogens. When the role of the pituitary axis was eliminated by hypophysectomizing the spayed guinea pig both the eosinophils/mm.^{3} and the proportion of eosinophils in the granulocyte population rose to twice the pre-operation level.

Hypophysectomy of adult male and female guinea pigs has previously (3) been shown to eliminate the sex difference in eosinophils by virtue of a rise in these cells which was three times as great in the male as in the female. This difference can be understood in terms of the data in the present report: In the males, the effects of hypophysectomy were additive—the eosinopenic influences of both adrenal cortical and gonadal hormones were reduced, but in the hypophysectomized female the reduction in the eosinopenic effect of adrenal cortical hormones was to some degree counterbalanced by a concomitant reduction in the eosinophilic effect of gonadal hormones. Calculations from data reported elsewhere (3) show that when females with intact ovaries were hypophysectomized the eosinophils/mm.^{3} and eosinophils/1000 polymorphonuclear leucocytes increased 62.8% and 71.8%, respectively, which is roughly half as great as the increase reported here for the spayed-hypophysectomized female. It must be noted, however, that until similar studies are done on the spayed-adrenalectomized guinea pig, the possible role of other pituitary factors cannot be ignored.

There was evidence in the present investigation that in addition to its eosinophilic effect estradiol will, under certain circumstances, induce an eosinopenia. The first suggestion of this effect occurred during an exploratory phase of the work when 8 guinea pigs spayed 4 months were injected with 20 μg. of estradiol daily for 2 weeks. Two days after the last injection there were only 52 ± 9.8 eosinophils/mm.^{3} of blood, a fall of 52.7% from pre-injection levels.

The meaning of this eosinopenia is clearer when we examine the later experiments in which estradiol was injected for shorter periods (Table 2). In analyzing these, special attention is given to the change in eosinophils/1000 polymorphonuclear leucocytes because there was a very significant increase in the latter cells. This may well have been a nonspecific effect of the injection procedure as the rate of disappearance in the spayed animal after the termination of injections was quite uniform (-20% between the 1st and 5th day; and -21% between the 5th and 8th day after treatment), although the persistence of an open vaginal closure membrane for from 7 to 10 days after the last injection indicated sustained high levels of estradiol. Five days after the last injection of estradiol (when the vaginal membrane was open in all animals) the eosinophils/1000 polymorphonuclear leucocytes had fallen to pre-injection levels in the spayed guinea pig but were still more than 100% above control levels in the spayed-hypophysectomized animal.

These results suggested an eosinopenic influence in the spayed guinea pig, presumed due to an effect of estradiol on the pituitary-adrenal axis since it was absent in the spayed animal which was hypophysectomized. Numerous reports indicate that estrogens do, indeed, stimulate the adrenal cortex by way of the pituitary-adrenal axis and in the guinea pig, in particular, Zondek and Burstein (9) have shown that estrogens mobilized ACTH causing enhanced excretion of urinary corticoids. Thus, estradiol appears to have a dual action: 1) an eosinophilic effect and 2) after longer treatment, an indirect eosinopenic effect produced, presumably, via the pituitary-adrenal axis.

Spayed-hypophysectomized females injected with testosterone manifested so marked

an eosinopenia that, despite the initial high level of eosinophils in the uninjected controls, there were too few cells even 8 days after the last injection to permit an accurate determination of their number. One animal had 94 cell/mm.^{3} of blood one day after the last injection, all other counts fell within a range from 0 to 30 cells. Clearly, testosterone does not produce its eosinopenic effect by way of the pituitary-adrenal axis and, except for the work of Zizine, Simpson and Evans (10) on the rat, the weight of evidence in the recent literature (11) indicates that it does not act directly on the adrenal gland. It may, therefore, be assumed that the effect of testosterone on the level of circulating eosinophils is not due to a release of adrenal cortical hormones.

SUMMARY

Eosinophil levels in the adult guinea pig, which are higher in the normal female than in the male, have been shown to be enhanced by estrogens: 1) Eosinophils in the normal female attained adult levels when the animals were about three months old. 2) Females ovariectomized before sexual maturity maintained pre-pubertal levels of eosinophils even six months after spaying. 3) Injections of 20 μg. of estradiol for five days in the spayed female increased eosinophils to above normal adult levels.

An eosinopenic effect of estradiol, which appeared later than the eosinophilic effect of the hormone, was demonstrated in the spayed female but was absent when the spayed animal was hypophysectomized. The reasons for assuming that this indirect eosinopenic action of estradiol occurs via the pituitary-adrenal axis are discussed.

The eosinopenia induced by testosterone was shown to be independent of the pituitary-adrenal axis since it occurred in the spayed-hypophysectomized female.

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INDUCTION OF MULTIPLE OVULATION IN THE RHESUS MONKEY (*MACACA MULATTA*)[1]

Although a number of follicles begin to develop during the early half of the sexual cycle in the primate, with few exceptions (e.g. marmoset) no more than one follicle expells its ovum. The rhesus monkey matures one ovum each menstrual cycle and twinning with nonidentical offspring occurs no more often than in man. The number of growing follicles can be increased easily in infantile, prepubertal and adult monkeys by the injection of the appropriate gonadotrophic fraction made from either sheep or monkey pituitary glands. Ovaries weighing less than 0.1 gm. may reach 4 gm., with follicles increasing from 1 to 6 mm. in diameter, after treatment with a gonadotrophin for five or six days. Supplements of pituitary interstitial cell stimulating hormone (ICSH) or human chorionic gonadotrophin (HCG) can readily induce luteinization. However, it has been more difficult to achieve the effective conditions for ovulation such as timing, amounts and relative proportions of the necessary hormonal components. In other animals when ovulation has occurred after treatment with gonadotrophins the number of ova discharged has been in excess of normal (superovulation); the same appears to be true in primates.

Figure 1 shows corpora lutea formed after multiple ovulation induced in adult monkey Mm 884 ($6\frac{1}{2}$ years; 5,180 gm.). The follicle stimulating fraction[2] made from pituitary glands of monkeys, *Macaca mulatta*, (MmPG) was injected at 2.5 mg. doses, twice daily, subcutaneously, for five days beginning the sixth day of the menstrual cycle. On the ensuing four days the follicle stimulating fraction was reduced to 1.5 mg. and mixed at each injection with 600 I.U. of HCG.[3] One ovary when removed 36 hours after the last injection (16th day of cycle) weighed 3.879 gm. and contained, in addition to large follicles, 12 corpora lutea estimated to be six days old.[4] All of the corpora lutea had rupture points indicating ovulations, which must have occurred soon after the addition of HCG. Nine days after a repeat series the other ovary weighed 1.42 gm. Large follicles were no longer present and the corpora lutea were consolidated, with repair of the torn surface at the sites of ovulation.

The other monkeys, Mm 812 and Mm 878, received the same daily dosage of the two hormones as outlined above, but with injections beginning on the fifth day of the cycle. The left ovary was removed from Mm 812 (9 years, 6,850 gm.) on the 15th day of the cycle. It weighed 2.561 gm. and showed three open stigmata. On histological examination

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[2] *Macaca mulatta* pituitaries were obtained through the courtesy of the Cutter Laboratories. The anterior lobes were dissected within 2 hours after death of the animals, and frozen immediately; later they were counted, pooled, weighed and lyophilized. Forty percent ethyl alcohol extracts were made and the soluble material was again dried. The potency of the preparation in terms of units of FSH and ICSH (MED for reinstatement of follicular growth and repair of the interstitial cells in the immature hypophysectomized rat) was 0.4 mg. given subcutaneously.

[3] The HCG was kindly supplied by Parke, Davis and Co.

[4] The ages of the corpora lutea were determined by comparison of the structure with those in ovaries removed from normal monkeys on known days of the cycle, also by comparison with Corner's figures in his account of the development of the corpus luteum in the macaque (1).

the corpora lutea were estimated to be from one to four days old, indicating that ovulation had, as in the first animal, begun soon after administration of the luteinizing agent. At autopsy seven days later the right ovary was found embedded in adhesions resulting from the raw surface incident to the multiple ovulation. The uterus held an actively secreting endometrium indicating one or more functional corpora lutea. Mm 878 (5 years, 5,095 gm.), was also examined 36 hours after the final injection, on the 14th day of the cycle. The laparatomy exposed a stimulated ovary, weighing 1.417 gm. The sites of recent ovulations appeared as oozing craters 3 to 4 mm. in diameter, rimmed with the everted

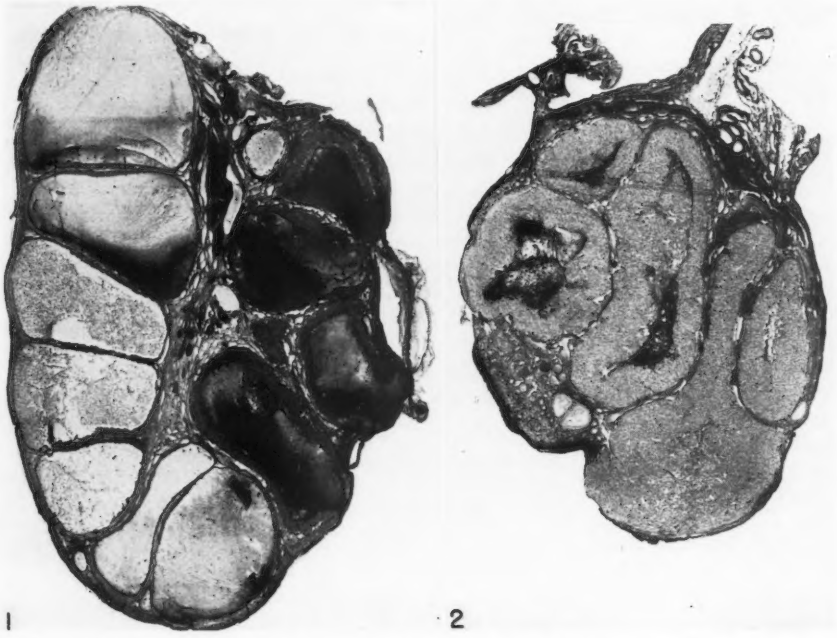

FIG. 1. Mm 884, adult; ovary removed 36 hours after injections, 16th day of menstrual cycle; 4 corpora formed after multiple ovulation, two with open stigmata, shown.

FIG. 2. Mm 878, adult; ovary removed 8 days after injections, 20th day of menstrual cycle; parts of 5 of the 9 induced corpora lutea are shown.

newly luteinized follicle walls. Six days later, on the 20th day of the cycle, the contralateral ovary (0.686 gm.) was removed (Fig. 2). Eight corpora lutea were present, in 5 of which ovulation sites could be identified. By this time the craters had been filled in with luteal tissue and the corpora lutea had become solid structures with the points of rupture still indicated by mushroom-like protuberances.

MmPG has been injected in seven other adult monkeys. However, in five no luteinizing hormone was given terminally and the remaining two animals received only $\frac{1}{3}$ and $\frac{1}{6}$ the effective amount of luteinizing hormone, and no ovulation occurred.

A prepubertal monkey born in the laboratory, Mm 887 (1 yr., 9 mo.; 3,120 gm.) showing no menarchal activity (vaginal desquamation, sex skin swelling or breast development) responded to an identical gonadotrophic hormone treatment with ovulation and the formation of multiple corpora lutea.

Although ovulation followed soon after the addition of HCG to the MmPG treat-

ment, its luteinizing action cannot be said to have been essential in precipitating ovulation. Chorionic hormone in some non-primate species is known to augment the follicle stimulating capacity of pituitary extracts. Alteration of the vascularity of the gonad should also be considered and the fact kept in mind that the monkey's own pituitary gland is present. The primate source of the pituitary substance may account for a particular efficacy at the immediate time of ovulation. The recent successful use of a primate growth hormone (2, 3) in monkeys has been interpreted as due to species specificity. However, in addition to the experiments herein reported, it has been possible to induce ovulation, but not consistently, in both prepubertal and adult rhesus monkeys using sheep pituitary preparations. Induced ovulation in the rhesus monkey, admittedly difficult to repeat, has been reported by others (4) long before the primate pituitary preparations were available.

SUMMARY

Multiple ovulation has been induced in the immature and adult rhesus monkey, *Macaca mulatta*, using a pituitary follicle stimulating substance prepared from rhesus pituitaries followed by a mixture of this fraction with human gonadotrophin. The primate source of the pituitary substance may account for the repeatedly successful experimental ovulations; however, ovulation has been observed occasionally following the use of sheep pituitary fractions as reported by earlier workers.

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CONCERNING THE PROGESTATIONAL ACTIVITY OF 21-HALOGENATED PROGESTERONES[1]

Recently, Tannhauser *et al.* (1) reported the synthesis of 21-fluoroprogesterone and the interesting finding that this substance possessed two to four times the luteoid activity of progesterone when assayed in the Clauberg test. Previously, Fried *et al.* (2, 3, 4, 5) had discovered that by suitable halogen substitution in position 9α of the steroid nucleus the corticoid activities of adrenal cortical hormones and their derivatives and of certain 11-ketoprogesterone derivatives were remarkably enhanced. (These findings were corroborated by results obtained in other laboratories; see for instance references

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6, 7, 8, 9.) A similar increase of corticoid activities was observed in the case of certain halogenations in position 12α (10, 11). Recently it has been reported (12, 13) that the introduction of a 9α-fluorine substituent increases not only the glucocorticoid but also the progestational activity of 11-ketoprogesterone.

In the well studied series of 9α-halogenated steroids it is established that the corticoid activity increases with the electro-negativity of the halogen substituent, the iodides being the least and the fluorides the most active representatives. Thus, the chlorides are highly active corticoids, their activities amounting in general to almost half of the activities of the corresponding fluorides. According to a preliminary report (13), the relationships in the 12α-halogenated series are similar although not as regular as in the 9α-halogenated series.

It therefore seemed of interest to investigate whether there existed similar relationships between the progestational activities of the 21-halo-derivatives of progesterone, especially whether the readily available 21-chloroprogesterone (14, 15) showed appreciable luteoid potency and how it compared in this respect with 21-fluoroprogesterone.

METHODS

The progestational activity of 21-chloroprogesterone was assayed by the McPhail test (16) at four dose levels, and the progestational activity of 21-fluoroprogesterone at 6 dose levels, as described previously (17); *in toto* there were used 35 animals. The results were compared with the corresponding activities of progesterone and 21-desoxycorticosterone (21-hydroxyprogesterone).

RESULTS AND DISCUSSION

The degree of progestational activity of the 21-halogenated progesterones may be compared with the previously found activities of progesterone and desoxycorticosterone acetate in Table 1.

TABLE 1. COMPARATIVE PROGESTATIONAL ACTIVITIES

Dose, in μ-moles	Activity			
	21-Chloroprogesterone, 1 μ-mole = 349.5 μg.	Desoxycorticosterone ac. 1 μ-mole = 372.5 μg.	Progesterone, 1 μ-mole = 314.4 μg.	21-Fluoroprogesterone, 1 μ-mole = 333.4 μg.
0	0,0,0 (Controls)			
0.125				+1, +1, +1, +1
0.17				0, +1, +1, +1
0.25				+1, +1, +1, +1
0.33				+3, +3, +3, +3
0.5			+3, +1, +1, +1	+3, +3, +4, +3
1.0	0, <1		+3, +3, +2, +2, +2, +4	+2, +4, +4
2.0	0, 0			
3.3		+2, +2, +2, +1, <1	+4, +4	
4.0	0, <1			
5.2		+4, +4, +4, +1		
6.0	0, <1			
8.0	0, 0			
10.0	0, <1	+4, +4, +4, +3		

In confirmation of the indications by Tannhauser *et al.* (1) we found 21-fluoroprogesterone to possess approximately three times the luteoid activity of progesterone.[2] On the other hand, even at doses of 3.5 mg. per animal (10 μ-moles) 21-chloroprogesterone showed no appreciable action on the uterus of the estrogen primed virgin rabbit. This is in contrast to a clear response following the administration of 0.5 μ-moles of progesterone and of 3.3 μ-moles of desoxycorticosterone acetate (17). Thus, 21-chloroprogesterone has less than one-sixtieth the activity of 21-fluoroprogesterone, less than one-twentieth the luteoid activity of progesterone and less than a third the activity of desoxycorticosterone acetate. The introduction of the 21-chlorine substituent, instead of increasing the luteoid activity, decreases it considerably. This behaviour is in sharp con-

[2] The buccal administration of approximately 20 μ-moles of 21-fluoroprogesterone also results in some luteoid action.

trast to the relationships in the 9α-halogenated series with respect to corticoid activities. The lack of parallelism is emphasized when it is considered that the 9α-hydroxylated derivatives show lower corticoid activities than the corresponding halides, whereas 21-hydroxyprogesterone is a far more potent luteoid than 21-chloroprogesterone. It therefore seems difficult to evoke, by analogy with the 9α-substituted series, the inductive effect of the 21-fluorine atom on the neighbouring carbonyl function as a cause for the enhanced progestational activity of 21-fluoroprogesterone. Neither can the lack of activity of 21-chloroprogesterone be explained by the assumption that in the 21-substituted series the steric requirements play a more important role than in the 9α-substituted series (i.e., that the 21-substituent has to be of a similar size as the original hydrogen atom), since substitution in position 21 with an hydroxy group leads to a more potent luteoid than substitution with a smaller chlorine atom.

After completion of this study, Fried and co-workers (18) reported that this lack of parallelism between the 9α-substituted series and the 21-substituted series was also true when the corticoid activities of 21-substituted cortisol analogues were compared to the corticoid activities of 9α-substituted cortisol derivatives; these authors found that the 21-chloro-derivative of the highly active 9α-fluorocortisol had little or no glucocorticoid activity.

SUMMARY

In contrast to the potent 21-fluoroprogesterone, 21-chloroprogesterone shows no appreciable luteoid activity; its activity is even less than that of 21-hydroxyprogesterone (desoxycorticosterone). The relationships between the progestational activities of 21-halogenated progesterones do not correspond to those encountered in the series of 9α-substituted corticoids where corticoid activity increases with the polarity of the 9α-substituent.

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THE PURIFICATION OF THYROXINE-BINDING PROTEIN BY ION-EXCHANGE RESINS[1]

During electrophoresis at pH 8.6, physiological concentrations of thyroxine in serum migrate in association with protein moieties localized in the zone between the α_1 and α_2 globulins (1–3). These moieties have been termed the "thyroxine-binding protein" of serum (TBP). The interactions of thyroxine with TBP and with secondary carriers of thyroxine (principally albumin) have been extensively studied in artificial plasma protein mixtures (4), and in sera obtained from normal individuals and from patients in a variety of abnormal states (4, 5, 6, 7, 8, 9).

Clarification of the physiological function and the physicochemical properties of TBP have largely awaited purification of the protein. Preliminary efforts in both directions have been reported previously (4, 10). It has been shown that TBP is concentrated in several globulin-containing fractions of serum, especially Fraction IV-6 (4), prepared by Method 6 of Cohn and his colleagues (11, 12). Fraction IV-6 has therefore been employed as a starting material for the attempts at further purification herein reported. Since the isoelectric point of TBP is lower than that of most of other other plasma proteins (13), it has been possible, by means of ion exchange techniques, to obtain preparations of the inter-alpha migrating protein which are very highly enriched and which demonstrate variable potencies in binding thyroxine. During this procedure, thyroxine is dissociated from the proteins of plasma by absorption on the resin, and can be subsequently eluted, in highly concentrated solution, free from protein.

METHODS

Experiments were performed at room temperature. Columns (20×1.5 cm.) of Dowex-1, X2, 200–400 mesh were equilibrated with acetate buffer, pH 4.5 of 0.05 ionic strength. Plasma proteins or Cohn fractions (IV-6,-IV-7, IV-9) were dissolved in similar buffer to a concentration of 4.5 gm.%, and the pH of the solution adjusted to 4.5 with dilute acetic acid. Solutions

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of protein were passed twice over the column at a flow rate of 15 ml./hour. The column was then washed with the original buffer until the optical density of the effluent at 280 mμ was negligible. Elution was performed with acetate buffers of progressively decreasing pH, and 0.10 ionic strength, in volumes equal to five times the dead space of the column. Eluates were extensively dialyzed against distilled water at 4° C, and were lyophilized. The dried powders were dissolved to a final concentration of 1.0 or 2.0 gm.% in distilled water, and their thyroxine-binding potency was assayed according to principles previously described (4).

RESULTS

Solutions of serum proteins, after exposure to the resin, demonstrated a decrease in TBP activity when compared to solutions of comparable concentration and pH which had not been passed over the column (Fig. 1). This finding indicated that TBP could be

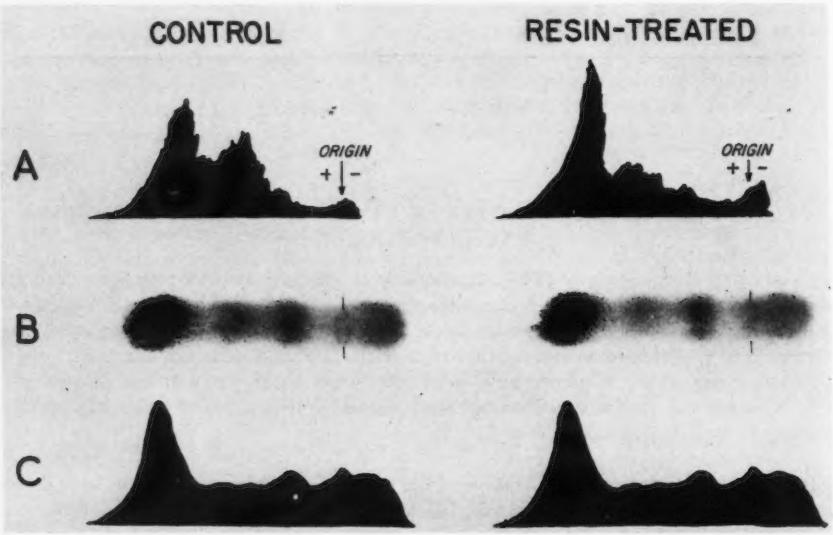

FIG. 1. Effect of Passage over Resin on the Activity of TBP in Serum: A. Radioactive scan of electrophoretogram. B. Brom-phenol-blue stain of serum proteins. C. Densitometric scan of brom-phenol-blue stain. During passage over the resin column, TBP activity is removed from serum without detectable change in the electrophoretic pattern of proteins.

partially separated from other proteins by adsorption onto the resin. A protein fraction having the electrophoretic mobility and thyroxine-binding properties of TBP was eluted by buffers of pH 4.0 in yields averaging approximately 0.3% of the starting material. Despite the addition to the original protein solutions of large amounts of I^{131}-labelled thyroxine,[2] only negligible amounts of radioactivity were found in those eluates which contained TBP.

When electrophoresed in pure solution at pH 8.6, the protein eluted at pH 4.0 bound thyroxine and migrated in the inter-alpha zone occupied by the TBP of concomitantly electrophoresed whole serum. However, successive batches of the protein thus obtained displayed variable potency in competing for thyroxine when electrophoresed in solutions containing mercaptalbumin (2.0 gm. %). Thyroxine-binding by one of the more potent

[2] Chromatographically pure, obtained from Abbott Laboratories, Oak Ridge, Tennessee.

preparations is illustrated in Figure 2. The anodal migration of the same preparation during electrophoresis at pH 4.5 is shown in Figure 3.

Ultracentrifugation of small amounts of a similarly active fraction revealed a single, but rather broad peak with a corrected S^{W}_{20} of 4.1.[3] Nitrogen/dry wt. ratio was found to be 10.2%.[4] Electrophoretograms stained by the PAS technique (14) were positive for carbohydrate.

Radioactive thyroxine added to solutions of starting material was almost entirely adsorbed during one passage over the column. Following the second passage, less than

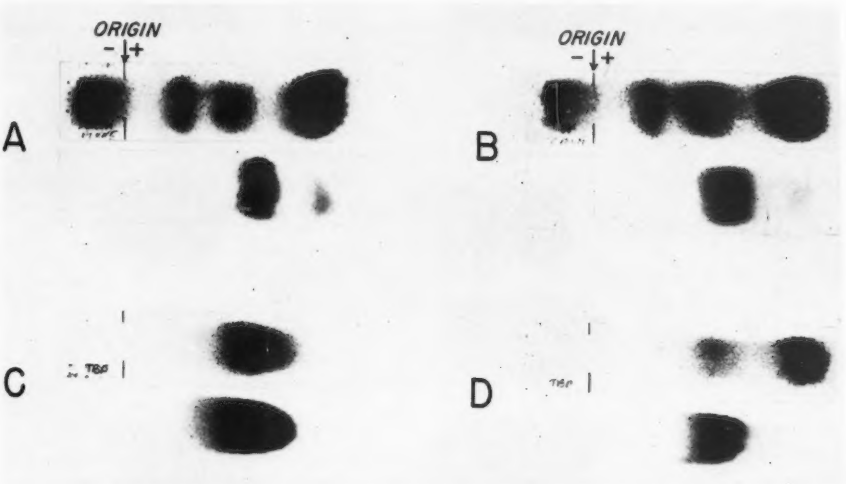

FIG. 2. Migration and Thyroxine-binding Characteristics of Column Eluate during Electrophoresis at pH 8.6. A. Serum. B. Serum plus column eluate (0.5 gm. %). C. Column eluate (2.0 gm. %). D. Column eluate (0.5 gm. %) plus mercaptalbumin (2.0 gm. %). In each group, upper pattern represents stain of proteins with brom-phenol-blue; lower pattern is a radioautograph demonstrating the distribution of added radio-thyroxine.

2% of added radioactivity could be detected in the effluent. Radioactivity remained adsorbed in a small band at the top of the column until acetate buffer of pH 1.5, 0.10 ionic strength, was employed, at which time the radioactivity was quantitatively eluted (98% recovered) in a small volume of effluent. No protein could be detected in the fractions which contained eluted radioiodine. The eluate retained its capacity to bind to protein, and, in combination with protein, all of the radioiodine remained undialyzable. Presumably, therefore, a significant degree of deiodination had not occurred. However, chromatographic identification of the eluted material was not obtained.

DISCUSSION

It is apparent that ion exchange chromatography at low pH can be employed to obtain markedly enriched, if not entirely purified, specimens of TBP. With regard to

[3] We are indebted to Dr. John L. Oncley of the Department of Biophysical Chemistry of Harvard Medical School for the ultracentrifugal analysis.

[4] Micro-analyses for nitrogen and dry weight were performed in the laboratory of Dr. Karl Ritter, Basle, Switzerland.

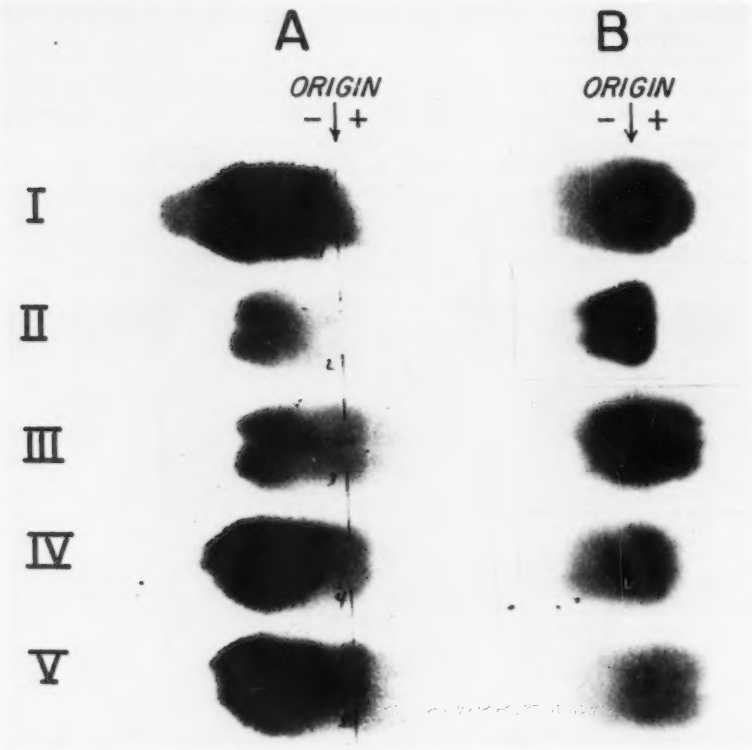

FIG. 3. Migration and Thyroxine-binding Characteristics of Column Eluate during Electrophoresis at pH 4.5. A. Brom-phenol-blue stain of proteins. B. Radioautograph of added radiothyroxine. I. Serum. II. Mercaptalbumin (2.0 gm. %). III. Mercaptalbumin (2.0 gm. %) plus column eluate (0.5 gm. %). IV. Serum diluted 1/1 with normal saline. V. Serum diluated with saline plus column eluate (0.5 gm. %).

nitrogen/dry weight ratio, sedimentation constant, and positive PAS reaction, the TBP thus obtained appears to be quite similar to the α_2 glycoproteins isolated by Schmid (15) and the M_2 mucoproteins of Mehl, Golden and Winzler (16). Electrophoretic migration of TBP at pH 4.5 is also comparable to that of the M_2 mucoproteins (13). However, at pH 8.6, TBP migrates more rapidly than the α_2 glycoproteins. In addition, specimens of the M_2 mucoprotein, purified without the use of the metallic cations which interfere with thyroxine-binding by TBP (4), fail to bind the hormone (17). It seems likely, therefore, that TBP and the M_2 mucoproteins are not identical. However, it is to be anticipated that such acidic proteins, similar in properties to TBP, will be included among the proteins obtained by the ion exchange technique. Their yield relative to that of TBP will depend at least in part upon their relative concentrations in the starting material. In this regard, Cohn fraction IV-6 has consistently provided TBP specimens of greater purity than have other globulin-rich fractions of serum.

The cause of the variable loss of binding potency of TBP obtained during different runs over the resin is at present unclear. Loss of activity might be ascribed to the strong

electrostatic field to which the protein is exposed while on the resin, to the low pH's employed, or to removal of the bound moiety (i.e., thyroxine). Alternatively, it is possible that passage over the resin may at times result in the removal of a prosthetic group essential for thyroxine-binding activity. Similar alteration in the physical properties of a protein following passage over a strong anion exchanger, and reversal following restoration of a presumed prosthetic group, have been previously reported (18, 19). These possibilities are currently being investigated in an effort to define conditions under which a more consistently potent TBP can be separated.

Among the interesting properties of the resin employed is its capacity to dissociate thyroxine from the proteins of plasma. Adsorbed thyroxine is retained on the resin while all demonstrable proteins are eluted, and the free hormone can then be eluted by further reduction in pH. The capacity of the resin is such that thyroxine from large amounts of serum can be concentrated in this manner, free from most other constituents of plasma. Provided that the behavior of the resin toward other intermediates of iodine metabolism can be shown to be comparable to its behavior toward thyroxine, the resin technique would appear to provide many advantages over conventional butanol extraction of serum for radioactive assay or chromatography.

SUMMARY

Fractions of the plasma proteins highly enriched in the Thyroxine-Binding Protein (TBP) have been obtained from Cohn Fractions of plasma by means of ion exchange techniques. Variable loss of binding activity occurs during this procedure.

During passage over the resin, the thyroxine of serum is adsorbed on the resin and can be eluted subsequently in highly concentrated form, free of protein, and suitable for chromatography.

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